UNIVERSITY OF ALBERTA

NANOPARTICULATE DELIVERY OF THERAPEUTIC VACCINE FOR CHRONIC HEPATITIS B

By

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“Be the change you want to see in this world”

- Mahatma Gandhi

“All that is not given is lost.”

- Rabindranath Tagore

This thesis is dedicated

in loving memory of my father.

Abstract

The focus of this dissertation is the evaluation of poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles as a delivery system for therapeutic vaccines for hepatitis B virus (HBV) chronic infection. PLGA formulations of HBV core antigen with or without an immunomodulator, monophosphoryl lipid A, were characterized with respect to size, surface morphology, antigen loading and release. Immune response studies in mice *in vivo* showed that co-delivery of core antigen and MPLA in nanoparticles elicited a strong T helper type 1 response with interferon-γ production by the activated T cells.

Pekin duck (*Anas platyrhynchos*) is an important preclinical animal model for hepatitis B. The duck MHC class II alpha (DRA) and invariant chain cDNA clones were sequenced and analyzed to facilitate the identification of antigen presenting cells (APCs). The deduced amino acid sequences of duck DRA and invariant chain cDNAs were homologous to human DRA and invariant chain, respectively. The transcripts of these two genes were found in the peripheral blood mononuclear cells (PBMCs) and in great abundance in the spleen.

Characterization of duck APCs was carried out with the adherent PBMCs. Duck monocytes/ macrophages were identified based on their physical and functional properties. Analysis of the parameters for particle uptake by the adherent PBMCs revealed that they were able to internalize PLGA nanoparticles (~600 nm) but not microparticles (~18 μm). Lipopolysaccharide treatment promoted the phagocytosis of PLGA nanoparticles and production of oxidative metabolites.

A preliminary study was performed in ducks chronically infected with duck hepatitis B virus (DHBV) to determine whether the immune responses elicited by PLGA nanoparticulate vaccines could intervene in a natural viral infection. The results showed the reduction of viral load in the vaccinated ducks was not significantly different from the control. However, a duck hepatitis B core-specific IgY antibody response was induced, suggesting that immune tolerance to the viral antigen can be broken by immunization with the nanoparticle formulation.

This research highlights the potential of PLGA nanoparticles as a vaccine delivery system for the induction of antiviral immune response and their use in studying immune-mediated viral clearance in DHBV model.

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TABLE OF CONTENTS

CHAPTER 1 1

INTRODUCTION 1

1.1 Introduction 2

1.2 Biology and life cycle of hepatitis B virus 3

1.3 Protective immune response in hepatitis B infection 5

1.3.1 Humoral immune response *5*

1.3.2 Cellular immune response *6*

1.3.2.1 Controlling virus replication 7

1.3.3 T helper responses *7*

1.4 Immune status during chronic hepatitis B infection 9

1.4.1 Immune tolerance *9*

1.4.2 Immune dysregulation *10*

1.5 Challenges in overcoming chronic hepatitis B 11

1.5.1 T cell hyporesponsiveness *11*

1.5.2 Th1/Th2 imbalance *12*

1.5.3 Liver tolerogenic microenvironment *13*

1.6 Restoration of immune activity – antiviral therapy 14

1.6.1 Current antiviral drug therapies for chronic hepatitis B *14*

1.6.2 Limitations of current antiviral therapies *15*

1.6.3 Supporting evidence for immunotherapy *16*

1.7 Design of therapeutic vaccines 17

1.7.1 Therapeutic vaccine for chronic hepatitis B *17*

1.7.2 Hepatitis B core antigen *18*

1.7.3 Antigen delivery systems *19*

1.7.4 PLGA nanoparticles as an antigen delivery system *19*

1.7.5 Factors regulating the induced immune response *20*

1.7.6 Size and hydrophobicity of the particles *20*

1.7.7 Antigen release profile *22*

1.7.8 Importance of adjuvants in the vaccine formulations *23*

1.7.9 Monophosphoryl lipid A *23*
1.7.9.1 Immunopotentiating effects 23
1.7.9.2 Modulation of immune response 24
1.8. ANIMAL MODELS FOR THE STUDY OF HEPATITIS B 25
1.9 DUCK IMMUNE SYSTEM 26
1.9.1 Duck antigen presenting cells *26*
1.9.2 A genomic approach to duck immune gene discovery *27*
1.10 DUCK HEPATITIS B VIRUS MODEL 27
1.11 RESEARCH PROJECT 28
1.11.1 Hypotheses *29*
1.11.2 Objectives *29*
1.12 REFERENCES 30

CHAPTER 2 56

ENHANCEMENT OF T HELPER TYPE 1 IMMUNE RESPONSE AGAINST HEPATITIS B CORE ANTIGEN BY POLY(D,L-LACTIC-CO-GLYCOLIC ACID) NANOPARTICLE VACCINE DELIVERY 56

2.1 INTRODUCTION 57
2.2 MATERIALS AND METHODS 59
2.2.1 Nanoparticles preparation *59*
2.2.2 Estimation of protein loading in nanoparticles *60*
2.2.3 Analysis of MPLA content in nanoparticles *60*
2.2.4 Surface morphology of nanoparticles *61*
2.2.5 Measurement of particle sizes *61*
2.2.6 In vitro release study of ^{125}*I-radiolabeled HBcAg-loaded nanoparticles* *61*
2.2.7 Immunizations *62*
2.2.8 T cell proliferative assay *63*
2.2.9 Antibodies assays *63*
2.2.10 Cytokine assays *64*
2.2.11 Statistical analysis *65*
2.3. RESULTS 66

2.3.1 Particle characterization *66*
2.3.2 *In vitro* protein release profile 67
2.3.3 Antigen-specific immune responses 67
2.3.3.1 T-Cell proliferative responses with a single immunization 67
2.3.3.2 T-cell proliferative responses with a booster immunization 68
2.3.3.3 Anti-HBcAg antibody responses 69
2.3.3.4 Cytokine Profile 69
2.4 DISCUSSION 70
2.5 REFERENCES 81
CHAPTER 3 89
IDENTIFICATION AND CHARACTERIZATION OF PEKIN DUCK MHC CLASS II DRA AND INVARIANT CHAIN GENES 89
3.1 INTRODUCTION 90
3.2 MATERIALS AND METHODS 91
3.2.1 Animal model *91*
3.2.2 Cloning of duck MHC class IIα and invariant chain *92*
3.2.2.1 cDNA library construction 92
3.2.2.2 DNA sequencing 92
3.2.2.3 Sequence analysis 93
3.2.3 Cell isolation and culture *93*
3.2.4 Reverse transcriptase-polymerase chain reaction (RT-PCR) *94*
3.2.5 Northern Blot Analysis *94*
3.2.6 Phylogenetic analysis *95*
3.3 RESULTS 95
3.3.1 Characterization of MHC DRA and Ii cDNA sequences *95*
3.3.1.1 Analysis of duck DRA cDNA and predicted polypeptide 95
3.3.1.2 Analysis of duck Ii cDNA and predicted polypeptide 97
3.3.2 Phylogenetic analysis *98*
3.3.3 Tissue distribution *99*

3.3.4 MHC class II DRα and the associated invariant chain mRNAs were expressed by macrophages *99*

3.4 Discussion 100

3.5 References 116

CHAPTER 4 122

IN VITRO CHARACTERIZATION OF THE ADHERENT PERIPHERAL BLOOD MONONUCLEAR CELLS OF PEKIN DUCKS, *ANAS PLATYRHYNCHOS* 122

4.1 INTRODUCTION 123

4.2 Materials and Methods 124

4.2.1 Animal model *124*

4.2.2 Isolation of monocytes from duck PBMCs *124*

4.2.3 Morphological and cytochemical analysis of cultured monocytes *125*

4.2.4 Characterization of PLGA particles *125*

4.2.4.1 Preparation of PLGA particles (microparticles versus nanoparticles) 125

4.2.4.2 Estimation of TMR-labeled dextran loading in particles 126

4.2.4.3 Measurement of particle sizes 126

4.2.5 In vitro phagocytosis *127*

4.2.5.1 Scanning Electron Microscopy (SEM) 127

4.2.5.2 Transmission Electron Microscopy (TEM) 127

4.2.5.3 Confocal Laser Scanning Microscopy (CSLM) 128

4.2.5.4 Fluorescence-activated cell sorter (FACS) analysis and isolation of phagocytic cells 129

4.2.6 Oxidative burst *130*

4.2.6.1 Greiss assay 130

4.2.7 MHC class II DRA and invariant chain expression by phagocytic cells *130*

4.3 Results 131

4.3.1 Particle characterization *131*

4.3.2 Morphological and cytochemical analysis of duck adherent PBMCs *131*

4.3.3 Uptake of PLGA particles by duck macrophages *132*

4.3.3.1 Electron microscopic analysis 132

4.3.3.2 Flow cytometric analysis 132
4.3.4 Kinetics of TMR-PLGA nanoparticle uptake *133*
4.3.5 Factors affecting the cellular uptake of PLGA particles: LPS, particle size, phagocytosis inhibitor and temperature *134*
4.3.6. MHC class II DRA and invariant chain expression by phagocytic duck cells *135*
4.3.7 Respiratory burst by the adherent PBMCs *135*
4.4 DISCUSSION 136
4.5 REFERENCES 150
CHAPTER 5 155
PLGA NANOPARTICLE DELIVERY OF DHBV VACCINE IN PEKIN DUCKS....155
5.1 INTRODUCTION 156
5.2 MATERIAL AND METHODS 158
5.2.1 Animals *158*
5.2.2 DHBV infection *158*
5.2.3 Vaccination Protocol *159*
5.2.4 Preparation of DHBcAg-loaded PLGA nanoparticles *159*
5.2.5 Measurement of particle sizes *160*
5.2.6 Estimation of protein loading in nanoparticles *160*
5.2.7 Serum DHBV DNA analysis - Real-time PCR assay *161*
5.2.8 Anti-DHBcAg IgY antibody production- ELISA *162*
5.2.9 Statistical analysis *162*
5.3 RESULTS 162
5.3.1 Particle characterization *162*
5.3.2 Antigen-specific immune responses *163*
5.3.2.1 Normal ducks 163
5.3.2.2 Congenitally DHBV-infected ducks 163
5.3.2.3 Experimentally DHBV-infected ducks 164
5.3.3 Virological outcome *164*
5.4 DISCUSSION 165

5.4 REFERENCES 173
CHAPTER 6 180
GENERAL DISCUSSION AND CONCLUSIONS 180
6.1 DISCUSSION 181
6.2 CONCLUSIONS 188
6.3 REFERENCES 189

LIST OF TABLES

Table 2-1. Levels of IFN-γ secreted by primed T cells co-cultured with either HBc peptide or HBcAg protein. Lymph node and spleen cell samples from mice that were immunized with various vaccine formulations in either a single or a booster immunization........80

Table 3-1. Oligonucleotide primers for MHC class II DRA and Ii. 115

LIST OF FIGURES

Figure 1-1. Life cycle of HBV 4

Figure 2-1. Scanning electron micrograph of surface morphology for (MPLA) nanoparticles (left panel), (HBcAg+MPLA) nanoparticles (middle panel) and (HBcAg) nanoparticles (right panel). 74

Figure 2-2. Release profile of ^{125}I-labelled HBcAg from PLGA nanoparticles in the presence or absence of MPLA. Samples were incubated at 37°C in phosphate buffer (pH 7.32) containing 0.01% Tween 20. Each point represents the mean ± SD of triplicate samples. 75

Figure 2-3. T-cell proliferative responses from the single immunization regimen. 76

Figure 2-4. T-cell proliferative responses from the booster immunization regimen 77

Figure 2-5. Anti-HBcAg humoral immune response (IgG) from the booster immunization groups. Mice were inoculated twice subcutaneously with one the following formulations: 1) HBcAg alone, 2) HBcAg + MPLA mixture and 3) (HBcAg + MPLA)-loaded nanoparticles. 78

Figure 2-6. Anti-HBcAg humoral immune response (IgM) from the booster immunization groups. Mice were inoculated twice subcutaneously with one the following formulations: 1) MPLA-loaded nanoparticles, 2) HBcAg alone, 3) HBcAg + MPLA mixture and 4) (HBcAg + MPLA)-loaded nanoparticles. The OD 405 absorbance reading represents the serum antibodies reactivity against HBcAg. 79

Figure 3-1. The nucleotide and the predicted amino acid sequences of Pekin duck MHC class II DRA. Borders of each domain were assigned based upon sequence homology between duck DRA and other DRA homologous sequences. 105

Figure 3-2. An alignment of the predicted amino acid sequence of Pekin duck MHC class II DRA with the DRA sequences of other species using CLUSTAL W 1.8 program. Identities are indicated by a dot; gaps by a dash. The small letter n and p indicate the positions of the predicted N-linked glycan attachment site and peptide-binding site, respectively. 106

Figure 3-3. Phylogenetic tree of deduced amino acid sequence of a full-length duck DRA sequences. Number at each node indicates the percentage of bootstrapping of a 1000 replications. Sequences included in the phylogenetic tree were obtained from the NCBI database at www.ncbi.nlm.nih.gov/. 107

Figure 3-4. The nucleotide and the predicted amino acid sequences of the Pekin duck invariant chain isoform, DuIi-2. Borders of each domain were assigned based upon sequence homology between duck other homologous Ii sequences. The additional nucleotide sequence, seen only in the Ii-2 isoform, is in bold. 108

Figure 3-5. An alignment of the predicted amino acid sequence of the DuIi-1 cDNA sequence with related sequences from the GenBank database using CLUSTAL W 1.8 program. Identities are indicated by a dot; gaps by a dash. An arrow indicates the position of the alternatively spliced exon 6b. 109

Figure 3-6. Alignment of the thyroglobulin type-1 (Tg) domain of the duck Ii-2 and chicken Ii-2 invariant chain. Identities are shown in shaded boxes. 110

Figure 3-7. Phylogenetic tree of deduced amino acid sequence of a full-length duck Ii sequence. Number at each node indicates the percentage of bootstrapping of a 1000 replications. Sequences included in the phylogenetic tree were obtained from the NCBI database at www.ncbi.nlm.nih.gov/. 111

Figure 3-8. Tissue expression of duck MHC class II DRA and invariant chain transcripts. Northern blot data indicated transcript sizes of around ~1.2 kb for DRA, and ~1.7 kb the Ii-2 isoform and ~1.5 kb for the Ii-1 isoform. Total RNA (10 μg) isolated from liver, spleen, lung, kidney and intestine was loaded from left to right. The original agarose gel stained with ethidium bromide demonstrated the relative amounts of RNA loaded in each lane. 112

Figure 3-9. RT-PCR analyses of duck DRA and Ii gene expression in mononuclear cells isolated from blood and spleen. Lane 1. Non-adherent mononuclear leukocytes from blood; Lane 2. Adherent cells (macrophages) derived from blood monocytes; Lane 3. Adherent cells (macrophages) stimulated with LPS (1μg/mL) for 24 hours; Lane 4. Non-adherent mononuclear leukocytes from spleen; Lane 5. Adherent cells (macrophages) derived from spleen cells. 113

Figure 3-10. Northern blot analysis of MHC class II DRα expression of duck macrophages at various time points after exposure to LPS (1μg/mL). 114

Figure 4-1. Scanning electron micrographs of PLGA nanoparticles (A) and PLGA microparticles (B)... 140

Figure 4-2. Light microscopic image of duck macrophages derived from blood monocytes x 63 under differential interference contrast (DIC) oil-immersion objective. 141

Figure 4-3 (A-C). Scanning electron micrograph of duck macrophages internalizing PLGA nanoparticles. Duck macrophages were fixed after an incubation of 6 h with PLGA nanoparticles. 142

Figure 4-4. Transmission electron micrographs of duck macrophages derived from blood monocytes. (A) Duck macrophages x 2400; (B) Duck macrophages that had internalized PLGA nanoparticles x 2400. (C)

Higher magnification of duck macrophages containing PLGA nanoparticles x 3600. Arrows indicated PLGA nanoparticles. 143

Figure 4-5. (A) Forward and side scatter dot plot (FSC/SSC) of duck macrophages derived from blood monocyte. (B) Dot plot (FSC/SSC) diagram of macrophages that have ingested TMR-PLGA nanoparticles. (C) Dot blot representing the TMR-PLGA nanoparticles. (D) Histogram of 10^4 events showing the fluorescence background from duck macrophages (No gate). (E) Histogram showing the overall fluorescent activity of macrophages after incubation with TMR-PLGA nanoparticles (No gate). The peak with higher fluorescence intensity (FL-2) represents the macrophage cell population that have ingested TMR-PLGA nanoparticles. 144

Figure 4-6. Confocal laser scanning micrographs of duck macrophages incubated with TMR-loaded PLGA nanoparticles over time in the absence of LPS (A-E) and in the presence of LPS (F-J). Image of duck cells after 24 hours incubation with large PLGA microparticles (K). 145

Figure 4-7. Flow cytometric analysis of the phagocytic response of duck macrophages to LPS, cytochalasin B or at low temperature. Duck macrophages were incubated with TMR-PLGA nanoparticles for 24 h in the presence or absence of LPS, cytochalasin B or at 4 ºC. 146

Figure 4-8. Expression of MHC class II DRα and invariant chain were determined from the isolated phagocytic cells. Lane 1: Phagocytic cells; Lane 2: LPS-treated phagocytic cells. The β-actin served as a loading control. 147

Figure 4-9. Effects of LPS on triggering nitrite (NO_2^-) by duck macrophages. After 48 hours incubation, 50 μL of culture fluid was removed and assessed for NO_2^- concentration. 148

Figure 4-10. Time course of nitrite (NO_2^-) production by activated duck macrophages. At zero time point, 500 ng/mL of LPS and 250 μM N^G-monomethyl-L-arginine (LPS + L-NMA) were added in the appropriate wells. At indicated incubation times, 50 μL of culture fluids were colleted and assessed for NO_2^- concentration. 149

Figure 5-1. Anti-DHBc IgY responses of normal Pekin ducks immunized with either (A) (DHBcAg+MPLA) nanoparticles, (B) DHBcAg or (C) PLGA nanoparticles control, on three occasions day 7, day 21 and day 35 (marked with arrows on the x-axis). Serum samples were collected seven days prior to the immunization (day 0) and seven days after each immunization. Each line represents the anti-DHBc response of an individual duck to the treatment over time. Serum samples were used at 1/50 dilution.... 168

Figure 5-2. Anti-DHBc IgY responses of congenitally DHBV-infected ducks immunized with either (A) (DHBcAg+MPLA) PLGA nanoparticles, (B) DHBcAg or (C) PLGA nanoparticles control, on three occasions day 7, day 21 and day 35 (marked with arrows on the x-axis). Serum samples were collected seven days prior to the immunizations (day 0) and seven days after each immunization. Each line represents the anti-DHBc response of an individual duck to the treatment over time. Serum samples were used at 1/50 dilution. 169

Figure 5-3. Anti-DHBc IgY response of experimentally DHBV-infected ducks immunized with (DHBcAg+MPLA) PLGA nanoparticles on three occasions day 7, day 21 and day 35 (marked with arrows on the x-axis). Serum samples were collected seven days prior to the immunizations (day 0) and seven days after each immunization. Each line represents the anti-DHBcAg response of an individual duck to the treatment over time. Serum samples were used at 1/50 dilution....... 170

Figure 5-4. Serological profiles of DHBV DNA of congenitally DHBV-infected ducks immunized with either (A) (DHBcAg+MPLA) PLGA nanoparticles, (B) DHBcAg or (C) PLGA nanoparticles control on day 7, day 21 and day 35 (marked with arrows on the x-axis). Serum samples were collected seven days prior to the immunization (day 0) and seven days after each immunization. 171

Figure 5-5. Serological profiles of DHBV DNA of experimentally DHBV-infected ducks immunized with either (A) (DHBcAg+MPLA) PLGA nanoparticles, (B) DHBcAg or (C) PLGA nanoparticles control on day 7, day 21 and day 35 (marked with arrows on the x-axis). Serum samples were collected seven days prior to the immunization (day 0) and seven days after each immunization. 172

LIST OF ABBREVIATIONS

aa	amino acid
ABTS	2.2'-azino-di-(3-ethylbenzthiazoline sulfonate)
ANCOVA	analysis of covariance
ANOVA	analysis of variance
APC	antigen presenting cell
Anti-DHBc	Anti-duck hepatitis B core antibody
Anti-DHBs	Anti-duck hepatitis B surface antibody
Anti-HBc	Anti-hepatitis B core antibody
Anti-HBe	Anti-hepatitis B e antibody
Anti-HBs	Anti-hepatitis B surface antibody
BSA	bovine serum albumin
BCA	bicinchoninic acid
cccDNA	covalently-closed circular deoxyribonucleic acid
CD40L	CD40 ligand
CLIP	class II associated invariant peptide
CLSM	confocal laser scanning microscopy
CMI	cell-mediated immunity
Con A	concanavalin A
CpG	cytosine-phosphate-guanosine
CPM	counts per minute
CT	cytoplasmic
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DHBV	duck hepatitis B virus
DHBsAg	duck hepatitis B surface antigen
DHBcAg	duck hepatitis B core antigen
DIC	differential interference contrast
DNA	deoxyribonucleic acid
DPM	decays per minute
EDTA	ethylendiaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter

FCS	fetal calf serum
FSC	forward scatter
FL	fluorescence
GSHV	ground squirrel hepatitis virus
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HEL	hen egg white lysozyme
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
HTL	helper T lymphocyte
IFN-α	interferon-alpha
IFN-γ	interferon-gamma
IgC	immunoglobulin domain constant region
IgG	immunoglobulin G
IgM	immunoglobulin M
IgY	immunoglobulin Y
IL-2	interleukin-2
IL-4	interleukin-4
IL-10	interleukin-10
IL-12	interleukin-12
IL-18	interleukin-18
Ii	invariant chain
LCMV	lymphocytic choriomeningitis virus
LIL	liver infiltrating lymphocyte
LPS	lipopolysaccharide
LSEC	liver sinusoidal endothelial cell
mAb	monoclonal antibody
mRNA	messenger ribonucleic acid
MFI	mean fluorescence intensity
MHC	major histocompatibility complex

MWt.	molecular weight
MPLA	monophosphoryl lipid A
NK	natural killer cell
NKT	natural killer T lymphocyte
nt	nucleotide
S-N-K	Student-Newman-Keuls
PAMP	pathogen-associated molecular pattern
PBML	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCS	photon correlation spectroscopy
PHA	phytohemagglutinin
PLGA	poly(D,L-lactic-co-glycolic acid)
PVA	poly(vinyl alcohol)
RPMI-10	RPMI media supplemented with 10% fetal calf serum
RT-PCR	reverse transcriptase polymerase chain reaction
s.c.	subcutaneous
SSC	side scatter
SEM	scanning electron microscopy
SI	stimulation index
SI_s	stimulation indices
TBAP	tetrabutylammonium dihydrogenphosphate
TCR	T-cell receptor
TEM	transmission electron microscopy
Tg	thyroglobulin
Th	T helper
TLR	toll-like receptor
TM	transmembrane
TMB	3,3',5,5'-tetramethylbenzidine
T_{reg}	regulatory T cell
TMR	tetramethylrhodamine
TNF-α	tumor necrosis factor-alpha
TPBS	phosphate buffered saline containing 0.05% (v/v) Tween
UV	ultraviolet
WHV	woodchuck hepatitis virus

w/o/w	water-in-oil-in-water

Amino acids

A	alanine
C	cysteine
D	aspartate
E	glutamate
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
W	tryptophan
V	valine
Y	tyrosine

Chapter 1

INTRODUCTION

1.1 Introduction

Hepatitis B virus (HBV) infection remains a major public health concern. It has been estimated more than 400 million people worldwide are persistently infected by HBV (1). Patients with chronic hepatitis B virus are at high risk of developing cirrhosis and hepatocellular carcinoma (HCC) (2). The current therapeutic treatment for chronic hepatitis B provides biochemical, virological and histological improvements but do not eliminate the virus and cure the disease. An alternative treatment strategy is urgently needed to resolve chronic HBV infection, and to prevent the development of progressive liver diseases.

The host immune responses play a key role in HBV control. Robust HBV-specific immune responses are thought to be responsible for the resolution of the liver disease (3). Immunotherapy aimed at promoting effective antiviral immune responses may offer a new approach for the treatment of chronic hepatitis B. Previous therapeutic vaccine clinical trials for chronic hepatitis B have shown some promising results. However, further studies are needed to improve the overall efficacy of the therapeutic vaccines for widespread use.

A thorough understanding of the immunological mechanisms for the control of hepatitis B virus infection is important in the design of an appropriate and effective vaccine. Biodegradable and biocompatible polymeric particles have been evaluated over the past few years for their potential use as a vaccine delivery system. Adjuvants have long been recognized as important components in the vaccine formulations. This thesis evaluates poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles as vaccine and adjuvant delivery systems for the induction of cellular immune responses. The following chapter provides the rationale for this research and the mechanisms involved in generating effective cellular immune responses against chronic hepatitis B.

1.2 Biology and life cycle of hepatitis B virus

Human HBV is the prototype of the *Hepadnaviridae* family. Other members of this family include duck hepatitis B virus (DHBV), woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV) (4-6). All these hepatotropic viruses can cause acute and chronic liver disease in their respective hosts. They have similar genomic organization, antigen composition and replication strategies. DHBV and WHV have been used as a model system to study viral replication, inhibition and mode of transmission.

Hepatitis B virion has an outer envelope protein, which consists of the large, middle and small surface antigens (HBsAg). The inner nucleocapsid protein carrying the core protein (HBcAg) encloses the viral genome and polymerase (reviewed by Tiollais et al (7)). The hepatitis B viral genome is a relaxed circular, partially double-stranded DNA molecule. Upon entry into the hepatocyte, the hepatitis B virus uncoats the envelope protein and enters the cytoplasm (Figure 1-1). The viral DNA plus strand is repaired within the nucleocapsid. The nucleocapsid delivers and releases the viral genome into the nucleus, where the viral genome yields a stable covalently closed circular DNA (cccDNA) molecule. The cccDNA molecule serves as a transcriptional template for the production of viral RNAs. The viral mRNAs are transported to the cytoplasm, where they are translated into viral structural proteins and viral enzymes.

During viral replication, one of the viral RNA transcripts, known as the pregenomic RNA, is encapsidated by the nucleocapsid. Within the nucleocapsid, the pregenomic RNA is reverse transcribed into a DNA minus strand, which in turn serves as the template for the DNA plus strand. Some of these newly formed core particles are transported to the nucleus for amplifying the viral genome inside the infected cell. Other core particles are assembled in the endoplasmic reticulum, where they associated with viral envelope to form a complete virion. The virion is then transported through the Golgi body and secreted as an infectious virion to infect other susceptible cells.

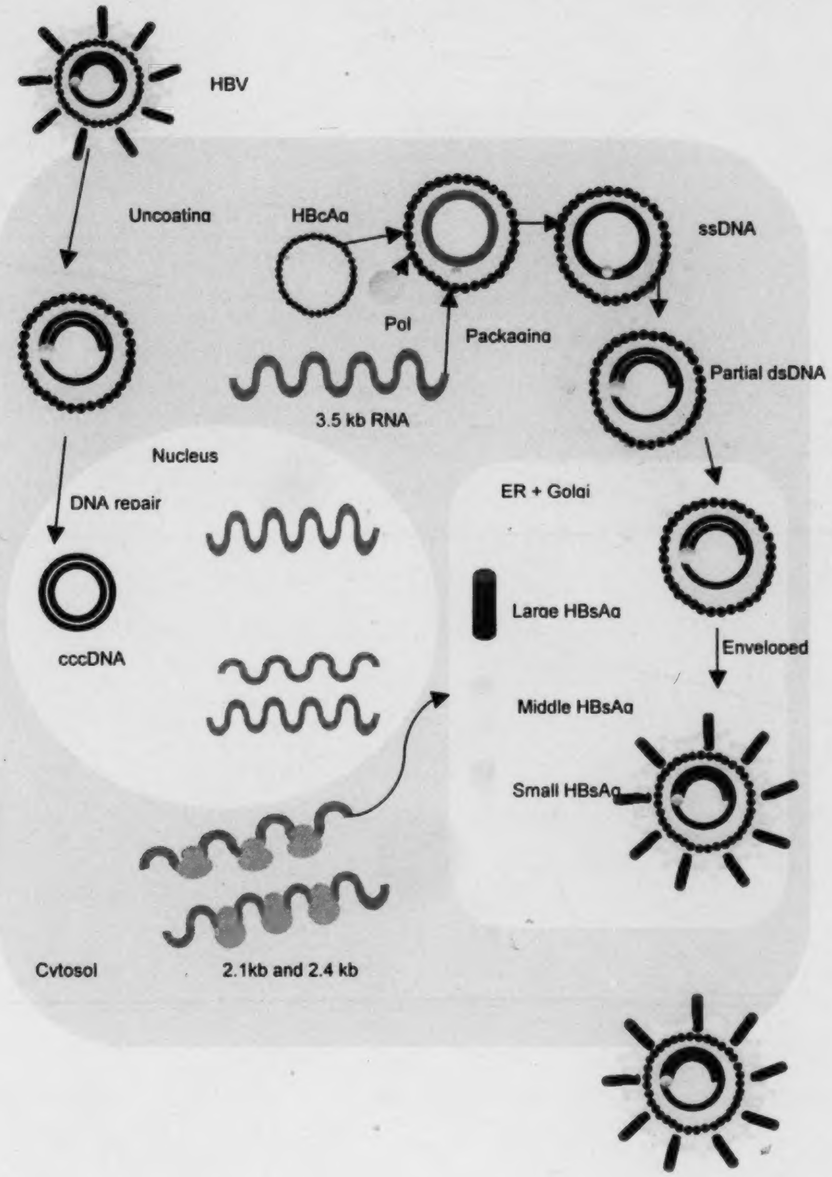
HBV
Uncoating
HBcAg
ssDNA
Pol
Packaging
Partial dsDNA
3.5 kb RNA
Nucleus
DNA repair
ER + Golgi
cccDNA
Large HBsAg
Enveloped
Middle HBsAg
Small HBsAg
Cytosol
2.1kb and 2.4 kb

Figure 1-1. Life cycle of HBV.

1.3 Protective immune response in hepatitis B infection

1.3.1 Humoral immune response

To control viral infection effectively, both cellular and humoral components of the immune system are essential. While cell-mediated immune response is critical in eliminating viruses once the infection has established, humoral immune response is needed to control viral load and prevent the spread of disease. The humoral immune response, which is adapted to the elimination of extracellular pathogens, is characterized by the production of large numbers of antibodies specific for an array of antigenic determinants on a foreign pathogen. It has been estimated that an average of 2.7×10^9 hepatitis B virus (HBV) DNA molecules/mL can be found in the bloodstream during an active hepatitis B chronic infection (8). As circulating hepatitis B viruses migrate to the liver, they can potentially infect more hepatocytes, initiate another replicative cycle and perpetuate the liver disease. An antibody can promote viral elimination by binding to the virus, forming opsonins or engaging Fc receptors on NK cells or macrophages to facilitate viral removal. Prophylactic vaccines generating neutralizing antibodies to hepatitis B surface antigen have been proven to be effective in protecting the susceptible host from hepatitis B infection (9). Hepatitis B immune globulin administered to infants born to HBV-infected mothers or patients receiving liver transplants, was able to prevent HBV infection (10, 11). A recent study reported that rapid production of neutralizing antibody was important to limit infection in the liver, contributing to the eventual transient outcome of the hepadnavirus infection (12). The appearance of (hepatitis B virus surface antigen) antibodies (Anti-HBsAg) was often associated with viral clearance, and has been used as an indicator for clinical recovery and the development of immunity to hepatitis B virus (13). Therefore, not only were neutralizing antibodies (Anti-HBsAg) vital for limiting the spread of virus, they were also necessary to protect the host from reinfection and to provide long-term immune control of HBV.

1.3.2 Cellular immune response

The recovery of a self-limited acute hepatitis B infection was associated with the production of vigorous polyclonal virus-specific T cell responses (14-17). Cumulative evidence has shown that $CD8^+$ T cells were the main effector cells responsible for viral clearance during acute HBV infection. Viral clearance was mediated by both noncytolytic and cytolytic effector functions of the $CD8^+$ T-cell response (18, 19). Adoptive transfer of activated cytotoxic T lymphocyte (CTL) from perforin-deficient (20) and Fas ligand-deficient mice (21) was still able to eliminate HBV transcript and antigen expression in the absence of cytolysis in the HBV transgenic mouse model. The non-cytolytic inhibition of both HBV transcription and replication was shown to be mediated by CTL-induced interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) (22, 23).

Similar non-cytolytic viral inhibition mechanisms have also been observed in activated intrahepatic natural killer (NK) cells and natural killer T (NKT) cells (24). Studies in chimpanzees and humans revealed that loss of HBV occurred before the peak of intrahepatic T cells and the occurrence of significant liver injury (25, 26). Therefore, in addition to $CD8^+$ T cells (18), other intrahepatic immune cell populations such as macrophages, NK or NKT cells may also play key roles for the early non-cytolytic antiviral immune responses (27).

Based on the study of resolution of transient woodchuck hepatitis B virus (WHV) infection, it has been proposed that the recovery process follows a sequential step-wise process (28). At the beginning of viral infection during the incubation phase, an early cytokine-mediated non-cytolytic process is responsible for reducing substantial amount of covalently closed circular deoxyribonucleic acid (cccDNA) molecules in the infected cells. This is followed by a cytolytic step in which CTLs induce apoptosis in the infected cells and eliminate the remaining cccDNA-containing cells. Lastly, a complete removal of the virus from the body is achieved by a regeneration of hepatocytes, which is necessary to remove any residual cccDNA in the hepatocytes. Studies of human hepatitis B viral clearance appear to support this sequential viral clearance model. They showed the kinetics of HBV DNA clearance followed a two or three phase decay pattern. During the first HBV clearance phase, HBV DNA has a mean half-life ($t_{1/2}$) of 3.7±1.2 days. The rate of HBV DNA loss in this phase was similar to the ($t_{1/2}$) observed in the noncytolytic clearance of cccDNA for duck hepatitis B virus (29). The final viral clearance phase involved the loss of infected hepatocytes and regeneration of uninfected cells occurred at a variable rate of ($t_{1/2}$ of 4.8 to 284 d) (30).

1.3.2.1 Controlling virus replication

Traces of the cccDNA have been shown to persist indefinitely at a very low level after complete clinical recovery from hepatitis B (19, 31). Long after the termination of acute hepatitis, asymptomatic mothers were still able to transmit pathogenic hepatitis B virus to their offspring (32). Using a sensitive PCR method, it was found that HBV DNA was detectable in the blood sample of convalescent patients (33). Despite the presence of serum antibodies and HBV-specific CTL at acute-stage, traces of HBV persist for years following clinical recovery (34). Moreover, vigorous antiviral T cell responses, comparable to that observed in the acute stage of infection, were maintained for decades after resolution of HBV disease. These results suggest hepatitis B virus once established in hosts, persists as an occult infection. Long-term maintenance of the T cell response was critical not only for keeping the virus under control but also protecting the host from viral reinfection (35, 36).

1.3.3 T helper responses

It is well established that $CD4^+$ T helper (Th) cells play a central role in regulating immune responses. Although $CD4^+$ T cell help was dispensable for priming $CD8^+$ T cell responses to infectious agents, many studies have shown that $CD4^+$ T help was necessary for the maintenance of a memory $CD8^+$ T cell population to mediate immunoprotective effect against reinfection (37-40). $CD4^+$ T cell depletion during the priming phase can lead to reduced memory $CD8^+$ T cell immune responses to reinfection (37). Furthermore, $CD4^+$ T cell memory cell depletion in immune animals before pathogen re-challenge also resulted in persistent infection rather than clearance (41). Recent adoptive transfer experiments in major histocompatibility complex (MHC) class II-deficient mice revealed that even though the formation of memory cell precursors did not require CD4$^+$ T cell help, CD4$^+$ T cell was crucial in maintenance of the quantity and the function of memory CD8$^+$ T cells after their development (42), which highlights the importance of CD4$^+$ and CD8$^+$ T cell collaboration in controlling persistent infections. These findings were supported by other studies demonstrating that the function of CD4$^+$ T cells were to augment the magnitude and promote survival of CD8$^+$ T cell responses, rather than programming functional properties (43).

The nature of this help in maintaining the memory CD8$^+$ T cell pool is largely unknown but it has been demonstrated that CD40-CD40L interaction is not required to mediate the effect (44). IL-15 and IL-7 have been shown to promote the proliferation and survival of CD8$^+$ memory T cells (45-48). IL-15 is produced constitutively by a wide variety of cells and tissues other than T cells (49, 50). Innate immune cells such as macrophages can produce a high level of IL-15 during inflammation (51). Although it has not been illustrated whether CD4$^+$ T helper cells are required for the induction of CD8$^+$ CTL cell against HBV during acute infection, the presence of strong and polyclonal and multispecific HBV-specific CD4$^+$ Th cells, in particularly core-specific CD4$^+$ Th cells, were often associated with the clearance of HBV during acute infection (52, 53). It has been suggested that the activation of a core-specific CD4$^+$ T cell response is essential for efficient control of viremia and a prerequisite for viral elimination. Adoptive transfer of HBV-specific Th1 cells in HBV-transgenic mice was sufficient to exert viral replication suppression in the liver (54). Given the fact that CD4$^+$ help is required to prolong the survival of functional CD8$^+$ T cells, it is conceivable that CD4$^+$ T cells play a fundamental role in supporting HBV immunity by maintaining long-lived HBV-specific CD8 T cells to control viral replication and prevent the development of chronic infection.

1.4 Immune status during chronic hepatitis B infection

Hepatitis B infection causes a spectrum of disease. While the majority of adults affected by hepatitis B virus recovered from acute hepatitis without serious sequelae, 5-10% develop a chronic infection with persistent liver inflammation (3). Although the underlying cellular/molecular basis for the development of chronic hepatitis B infection is not known, the clear immune dichotomy between acute and chronic HBV infection has led to the general view that the balance between the virus and host factors is likely the determinant for the outcome of infection. The deficiency of appropriate immunological responses to the virus and/or the exposure of overwhelming viral load during acute infection were strongly associated with the development of viral persistence (55, 56).

1.4.1 Immune tolerance

Both human and animal studies show that the age at which infection occurs plays a key role in determining the outcome of infection (55, 57). Neonates infected with HBV perinatally often failed to develop effective immune responses against the virus, probably due to the immune immaturity at the time of infection. These individuals became tolerant to the virus and remained as asymptomatic chronic healthy carriers (58, 59). Absence of acute-phase cellular immune activity was also associated with the development of chronic HBV infection in neonatal woodchuck (55). Immunosuppressive drugs suppressed T cell function and induced chronic disease, in otherwise a transient woodchuck hepatitis virus infection (60). Alteration of the outcome of DHBV infection outcome by modifying the host immune competent state has been demonstrated in duck hepatitis B virus infection (61). The immunological status of the host at the time of infection appears to have a significant role in determining the pattern or outcome of infection.

1.4.2 Immune dysregulation

A small percentage of the apparently immunocompetent individuals infected with HBV are unable to clear the virus. Although the major events that lead to the onset of chronic hepatitis B infection has not been fully elucidated, the observation from the experimental studies of other viruses (reviewed by Zinkernagel (62)), provide some clues to the development of chronic hepatitis B infection. It has long been recognized that viruses can devise various strategies to evade immune recognition and elimination (63). In addition, a high viral load contributing to the development of chronic infection has been observed in duck hepatitis B virus model (64, 65). Rapid and overwhelming infections cause a 'complete' induction of CTL. The initial vigorous T cell responses were quickly becoming clonally exhausted and resulted in the deletion of a large number of activated antiviral T cells, thus reducing the host's defenses against the invading virus and succumbing to a viral persistence state in an otherwise immunocompetent host (66-69). Similar events may also happen during the development of chronic hepatitis B infection, as few or absence of HBV-specific T cells was usually found in patients with chronic hepatitis B (3, 17, 70). Hence, an immediate control of viral replication via cytokine-mediated mechanism during early infection is likely to be critical in determining the final outcome of HBV infection, since overwhelming viral load appears to have a detrimental effect on the efficiency of T cell-mediated viral clearance.

HBV can also exert a nonspecific suppressive effect and render immune cells ineffective (71, 72). Impaired functional activities of dendritic cell (DC) have been reported in chronic HBV patients (73-75), which may account for their failure to elicit effective antiviral T-cell responses. Alternatively, high viral load has been shown to skew the immune response towards the Th2-type (76, 77) as it promotes the production of IL-4, IL-5 and IL-10 which support antibody response rather than cell-mediated immunity. Viral persistence in chronic HBV has been attributed to an imbalance in the Th1-Th2 arms of the immune response (78). Unfavourable Th2 polarization could profoundly influence the subsequent course of disease after infection and the susceptibility to persistent infection. Marked intrahepatic IFN-γ and TNF-α deficiencies at the acute stage have been associated with the progression to chronic infection in experimental woodchuck hepatitis virus infection (79-81). As viral replication was more likely to be controlled by intracellular inactivation mediated by cytokines rather than by hepatocyte death at the initial amplification stage (20), the capacity of the host to produce IFN-γ and TNF-α cytokine would certainly play a role in the establishment of the disease and the progression to chronic infection.

1.5 Challenges in overcoming chronic hepatitis B

1.5.1 T cell hyporesponsiveness

Weak or barely detectable virus-specific T immune responses were frequently found in patients with chronic HBV infection (17, 82). However, when they were detectable, the effector functions of these virus-specific T cells were found to be altered (71, 83). Peripheral tolerance mechanisms such as anergy or ignorance may partially explain the coexistence of high virus load and virus-specific immune responses. On the other hand, virus mutants that were not recognized by CTL could lead to viral escape, especially if the CTL response against that epitope was crucial for viral clearance during chronic HBV infection (84). CTL mutant epitopes that acted as antagonists and rendered T cells nonfunctional, had been identified from HBV chronically infected patients (85). A naturally occurring mutation within immunodominant epitopes of the HBV nucleocapsid that emerged during the course of chronic HBV infection has been shown to affect the antiviral $CD4^+$ T-lymphocyte reactivity (86). A general deficiency in helper T lymphocyte responses to immunogens has been observed in the population of patients with chronic hepatitis B (71), and whether or not virus mutation is related to such immune hyporesponsiveness has not been examined.

Other factors may also be involved in the development of T cell hyporesponsiveness in these patients. Long-term antigen exposure has been shown to be a critical factor for the hierarchical loss of $CD8^+$ T cell effector functions during chronic lymphocytic choriomeningitis virus (LCMV) infection and the subsequent deletion of T cells (87). Long-term defects in cytokine production, alteration in expression of cell surface L-selectin (CD62L) and significant changes in the epitope hierarchy of the LCMV-specific memory CD8$^+$ T cells were shown to arise from long-term exposure of CD8$^+$ T cells to antigen stimulation during chronic LCMV infection (88, 89). Dysfunctional envelop-specific CD8+ T cells were found in patients with chronic HBV infection (90, 91). Thus, prolonged exposure of antigen during chronic infection may also play a role in subverting virus-specific T cell functions. These additional obstacles that arise during chronic infection may further prevent the host from eliciting effective antiviral immune responses as the disease progresses.

1.5.2 Th1/Th2 imbalance

Th1 and Th2 are the subtypes of T helper cells, which have been defined on the basis of their cytokine secretion patterns. The decision of an activated T cell to differentiate into Th1 or Th2 is crucial, since it determines whether a cell-mediated or humoral immune response is triggered against the invading pathogen, which can profoundly influence disease outcome. Viral persistence in chronic HBV carriers was characterized by the production of low levels of IFN-γ, IL-2, IL-4 and IL-5 in response to viral antigens, a Th0-type like anti-HBV immune response (92, 93). This is in stark contrast to patients with self-limited acute hepatitis B, who produced a high level of IFN-γ against viral antigens and a predominantly Th1 immune response (93). The imbalance in the production of Th1/Th2 cytokines by HBV-specific T cells prohibited an efficient induction of Th1 cell and CTL, which was evident in chronic HBV carriers whose Th and CTL were quantitatively insufficient (94). The antigen presenting cells (APCs) and/or regulatory CD4$^+$ T lymphocytes in children with chronic hepatitis B infection were shown to preferentially produce IL-10 (95). Excessive IL-10 produced by peripheral blood mononuclear cells (PBMCs) and liver infiltrating lymphocytes have also been reported in patients with chronic HBV infections (96). IL-10 produced in these individuals may exert an anti-inflammatory and suppressive effect on the development of HBV-specific Th1 cells.

Interleukin-12 induction of Th1 cytokines was important for viral clearance in chronic hepatitis B (97). An overall defect in IL-12 and IFN-γ secretion have been observed in patients with chronic HBV (71). This may also bias the immune response unfavorably towards Th2-type and hamper the induction of effective antiviral immune responses.

1.5.3 Liver tolerogenic microenvironment

The local tissue microenvironment plays an essential role in determining the magnitude and the type of local immune responses. The unique anatomical structure and cytokine milieu in the liver create a tolerogenic environment for any antigen it encounters (reviewed by Crispe (98)). In particular the liver sinusoidal endothelial cells (LSECs), lining the hepatic sinusoids, can act as antigen presenting cells with a strong predisposition towards tolerance induction (99). Like Kupfer cells, LSEC are efficient in capturing and presenting antigen to T lymphocytes in the sinusoids. A recent study has demonstrated that LSEC expressed only a minimal MHC class II and undetectable levels of CD80, CD86, CD40 and CD11c (100). In the absence of co-stimulatory molecule expression, LSEC are poor stimulators for allogeneic or antigen-specific T cells activation. The lack of CD80 or CD86 expression on hepatocytes, bile ducts, Ito cells or LSEC has also been documented in human samples (101). Antigen acquired by LSEC and presented to T cells in the absence of costimulatory molecules can result in immune anergy and T cell tolerance (102). HBV antigens secreted in the blood stream can be captured and presented by LSEC, but whether viral antigen presentation by LSEC during chronic infection can lead to viral persistence has not been fully investigated.

Studies have shown that liver sinusoidal endothelial cells selectively suppress the expansion of IFN-γ-producing cells and promote the growth of IL-4-expressing Th2 cells, supporting the theory that liver has a general propensity to suppress cellular immune response (103). In addition, the hepatic microenvironment was rich in immunoregulatory mediators such as IL-10 and transforming growth factor (TGF)-β (104, 105). These immunosuppressive cytokines can inhibit production of the Th1 driving cytokine IL-12 from dendritic cells, suppress costimulatory molecule expression and maturation, and render dendritic cells ineffective as antigen presenting cells (106-108). Downregulation of the immunostimulatory effect of monocyte-derived dendritic cells was evident after their incubation with liver-conditioned media (104). Furthermore, an induction of regulatory T (T_{reg}) cells by immature DC has been proposed as a mechanism of tolerance (109, 110). The involvement of T_{reg} in maintaining chronic viral infection has been described in Friend retrovirus-infected mice (111) and in patients with chronic hepatitis C virus infection (112). A recent study showed T_{reg} cells play a role in the maintenance of viral persistence in hepatitis B (113). Whether or not T_{reg} cells are predominantly located in the liver to maintain tolerance is not known. But certainly, the overall suppressive milieu within liver can pose an additional barrier to overcoming the immune tolerance to chronic viral infection in the liver.

1.6 Restoration of immune activity – antiviral therapy

1.6.1 Current antiviral drug therapies for chronic hepatitis B

The dynamic process between the antiviral immune response and viral replication is likely to determine the disease outcome. A weak and impaired T cell response against HBV antigens during chronic infection contributes to the loss of viral control and the pathogenesis of the disease (17, 82) . Such immune hyporesponsiveness, however, was reversible in some patients. About 5-10% of chronically infected patients clear HBV DNA and HBeAg from serum spontaneously each year (114). This process was frequently preceded by an acute exacerbation of liver disease and accompanied by elevated virus-specific T cell frequencies and proliferative responses (115). The induction of strong HBV-specific CTL response was often involved in chronically infected patients who experienced a spontaneous or interferon-induced viral clearance (116), suggesting that an effective HBV-specific immune response could be potentially activated in chronic HBV patients. Although the basis for the spontaneous immune augmentation is unknown, a reduction of viral load in chronic HBV patients by lamivudine antiviral drug treatment is temporally associated with an enhancement of $CD4^+$ and $CD8^+$ T cell activities against HBV antigens (83, 117, 118). An increase of Th1 immune responses against HBV was attained by reducing viral burden with antiviral treatments (119). These studies suggest that a high viral load may be the major factor for T cell hyporesponsiveness; thereby lowering the viral load with a nucleoside analogue may recover T cell responsiveness and regain control over viral replication. However, the restoration of HBV-specific T cell reactivity with lamivudine was not durable, it peaked within 1-4 weeks and was followed by a progressive decline to the pretreatment level despite continual lamivudine treatment (120). The transient nature of such immune reconstitution indicates that an additional therapy is required at an early stage to sustain or strengthen the immune responses against the HBV virus, which may improve the durability of the T cell reactivity and complete the viral elimination process. Patients with chronic hepatitis B who responded to combination therapy with ribavirin and IFN-α, were able to mount a lasting HBV-specific Th1-type $CD4^+$ T-cell proliferative response, producing increased levels of IL-12 and IFN-γ and decreased levels of IL-10, that was still detectable one year after HBV DNA clearance (121).

1.6.2 Limitations of current antiviral therapies

Currently no effective treatments are available to treat chronic hepatitis B. IFN-α has been used as the first line therapy for chronic HBV infection since the 1970s (122). The response rate, defined as a sustained loss of viral replication and normalization of serum transaminase levels following the IFN-α antiviral monotherapy is only 20-30% (123). Nucleoside analog antiviral treatment such as lamivudine is used to reduce viral replication, liver inflammation and tissue injury (124). Cessation of drug treatments often results in virus rebound and relapses of viral infection (125). Prolonged therapy leads to the emergence of viral resistance (126-128) and eventually treatment failure. Most importantly, nucleoside analogue antiviral therapy does not result in viral elimination since it does not remove viral cccDNA, which is the source of viral infection and maintenance of viral replication.

1.6.3 Supporting evidence for immunotherapy

Immunomodulatory reagents such as IL-12, GM-CSF and thymosin α-1 have shown some encouraging results in suppressing viral replication in patients with chronic HBV (129-131). Many different strategies have been used to design specific therapeutic vaccines for the treatment of chronic HBV including protein, lipopeptide, DNA and dendritic cell-based vaccines. Most of these strategies have shown some success, in either breaking viral tolerance when tested in HBV-transgenic mice, or inducing vigorous cellular responses in normal healthy subjects. However, vaccination results within the chronically HBV infected patients were somewhat variable based on the few clinical studies reported so far.

A multi-center clinical controlled study indicated that recombinant protein vaccines containing either Pre S2/S or S may reduce HBV viral load in chronically infected hepatitis B infected patients(132). It has been shown previously in a pilot study that the reduction of viremia was associated with the induction of HBV-specific Th1 responses (133). However, the end results of the study showed that the rate of HBV DNA disappearance between the vaccinated and unvaccinated groups was not much different (132). A protein-based vaccine composed of pre-S1, pre-S2 and S antigenic components of the HBV was evaluated in 22 chronically HBV infected patients (134). It was found that the vaccine induced a significant HBsAg-specific T-cell proliferation. However, the cytokine profile (i.e. IL-5) obtained from the tested subject indicated a Th2 response rather than a Th1 response. No clear correlation was found between the strength of HBsAg proliferative response and the reduction of HBV DNA in this study.

A lipopeptide vaccine consisting of three covalently linked components: a CTL peptide epitope based on the HBcAg 18-27, a Th cell epitope from residues 830-843 of tetanus toxoid and two palmitic acid molecules as the lipids, was designed to induce HBV-specific CTL responses (135). The results of this pilot study revealed that HBV-specific T cell responses could be elicited in chronically infected patients with the designed lipopeptide vaccine. However, the induced CTL activity was relatively low compared to that observed during spontaneous HBV clearance and the CTL reactivity did not correlate with viral clearance.

Although the preliminary therapeutic vaccines studied so far lack clinical efficacy when administered to patients with chronic HBV infection, the results from these different vaccine trials confirmed that reduction of viremia and induction of antiviral cellular immune responses were both achievable by active specific immunization in the chronically HBV infected patient (132-134). Most importantly, these vaccination treatments were safe and well tolerated. In addition, these studies corroborated findings in other studies that antiviral T cells in chronically infected patients have not been completely deleted and therapeutic intervention may be useful in inducing their reactivity to mediate viral replication in the lamivudine treatment study (83). A more effective therapeutic vaccine is urgently needed to improve the efficacy of these experimental vaccines. A combination of lamivudine, IL-2 and HBV surface antigen vaccine has shown some promising results from a small clinical trial (136). Preliminary data indicated that such combination therapy induced antiviral immune responses that led to viral elimination in some chronic hepatitis B patients.

1.7 Design of therapeutic vaccines

1.7.1 Therapeutic vaccine for chronic hepatitis B

The common goal of vaccine treatment for chronic HBV is to slow or reverse the clinical course of the disease. Therapeutic vaccines for chronic HBV must be designed to generate sufficient quantity and quality of virus-specific T cells to overcome viral tolerance and mediate viral control. Hepatitis B core antigen is a promising vaccine candidate. PLGA nanoparticles are an efficient vaccine delivery system for targeting APC and enhancing immune responses to the incorporated antigen. Adjuvants are required to generate an effective immune response. They serve to activate the adaptive immune system, promote and up-regulate the antigen presenting activity of APC by inducing cytokine production and co-stimulatory molecule expression on APC. Therefore, it is essential to include adjuvants in a vaccine formulation to boost APC signalling to enhance immune responses. Monophosphoryl lipid A (MPLA), as an adjuvant and an immunomodulatory agent, can help mobilize and promote Th1 responses. In an attempt to develop better therapeutic vaccines for chronic hepatitis B, we propose using PLGA nanoparticles as a vaccine delivery system to deliver both hepatitis B core antigen and MPLA. Co-delivery of the antigenic and adjuvant stimuli to the immune system may enhance antigen uptake and presentation by APC, and provide an effective means to stimulate T-cell responses. Potent Th1 responses may also be induced by this vaccine formulation and offer potential clinical benefit for chronic HBV-infected patients.

1.7.2 Hepatitis B core antigen

Hepatitis B core antigen (HBcAg) has been described as the most vigorously recognized antigen for HLA class II restricted, $CD4^+$ T cells in self-limited acute HBV infection (17). The peak of the *ex vivo* $CD4^+$ T cell response to HBcAg coincided with the loss of serum HBeAg and HBsAg (52). The presence of high frequency of core -specific $CD8^+$ T cells was also associated with control of HBV infections (15). Adoptive transfer of HBcAg–specific T cells has been shown to provide intermolecular help for anti-envelope antibody production and resulted in successful HBsAg clearance and resolution of chronic infection (137). Overall, there is convincing evidence that hepatitis B core antigen (HBcAg)-specific T cell responses contribute to viral clearance and the resolution of the HBV disease (94, 138). Therapeutic vaccines aimed to stimulate core-specific T-cell responses may lead to viral control and the termination of the disease.

1.7.3 Antigen delivery systems

Subunit vaccines usually require more than a single immunization to induce adequate immune responses. The goals of early vaccine delivery systems were to have a controlled-release vaccine in a single dose formulation to reduce the frequency of parenteral immunizations (139), and to improve the stability of protein vaccine (140). Liposome, polymeric microparticles and implants have been formulated to release antigen in a continuous or pulsatile manner, thereby mimicking the repeated injections of conventional vaccination schedules. These vaccine delivery systems also served as adjuvants to the encapsulated antigens. In particular the immunopotentiating effects of the particulate vaccine delivery system to the associated antigen have been demonstrated in many animal studies. Several mechanisms of their adjuvant actions have been proposed: (i) the "depot" effect, allowing sustained-release of antigens and prolonging their interaction with the immune cells; (ii) targeting and facilitating antigen uptake into antigen presenting cells; (iii) altering the kinetics of antigen clearance; (iv) promoting antigen entry into lymph nodes.

1.7.4 PLGA nanoparticles as an antigen delivery system

Micro or nanoparticles prepared from PLGA polymer can be used as an antigen delivery system. The use of biodegradable and biocompatible poly(D,L-lactic-co-glycolic acid) (PLGA) polymeric material as vaccine carrier is favourable due to its long-history of safety and tolerability profile in human use. It has been used as suture material and controlled-release drug delivery device in humans. The immunopotentiating effects of PLGA particles to the encapsulated antigen have been demonstrated in various animal models (141-143). Moreover, the adjuvant effect of PLGA micro/nanoparticles has been shown to be superior to that of aluminum hydroxide (144) and Freund's complete adjuvant (145) and

PLGA microparticles were capable of generating effective CTL responses compared to other available adjuvants (146-148).

1.7.5 Factors regulating the induced immune response

The nature of the antigen, antigen dose and the route of immunization are distinctive factors in determining the magnitude and the type of immune response generated (149-151). When an antigen is formulated into a particulate vaccine delivery system, other factors including the physiochemical properties of the delivery vehicle become important with respect to its interaction with the antigen and the immune cells. For example, an antigen may interact with the vaccine carrier in a way that may alter the intrinsic nature of the antigen thereby its immunogenicity (152). In addition, antigen loading in vaccine carrier can dictate the antigen release profile (153) and the duration of immune responses (154). Antigens encapsulated in a vaccine carrier may change their distribution and clearance. Since the localization and migration of antigens are critical in controlling immunity and tolerance (77), vaccine formulation altering the fate of antigen in the body may have a profound effect on how the immune system interacts and responds to the encapsulated antigens.

Several physiochemical parameters of the vaccine delivery system are known to influence the induced immune responses. Notably, the size, hydrophobicity, porosity and degradation rate of the polymeric particles as vaccine carrier have been shown to influence the vaccine efficacy (155). While the size and hydrophobicity of particles mediated their impact on the immune system by controlling uptake by the antigen presenting cells (156-158), the surface morphology and the degradation rate of the polymeric particles were related to the pattern of the antigen release (159, 160), which were particularly important in shaping the development of humoral immune response (161, 162).

1.7.6 Size and hydrophobicity of the particles

Particle uptake by macrophages decreases as the size of the particles increases (157). Phagocytosis can also be increased by increasing the hydrophobicity of the polymer (158). Other investigators have found that the maximal phagocytosis of particles occurred with a hydrophobic surface and a size range between 1.0-2.0 microns (156). The delivery of staphylococcal enterotoxin B (SEB) toxoid via PLGA particles of 1 to 10 μm in diameter, induced an IgG antitoxin response which was approximately 500 times that seen with non-encapsulated toxoid when injected subcutaneously in mice. When mice were immunized with the same dose of toxoid in large particles which were between 10-110 μm in diameter, the induced IgG antitoxin response was only one-tenth that seen with the smaller particles at comparable time periods (163). Small particles (1-10 μm) were taken up by the APC and subsequently delivered to the draining lymph nodes. In contrast, particles of a size >10 μm were not phagocytosed by macrophages and remained localized at the site of injection. These results suggested that adjuvant effect of antigen-containing PLGA particles correlated with particle size, which was related to their abilities to be taken up and transferred to the draining lymph nodes by the antigen presenting cells (163). Similar observations have been made regarding cellular immune potentiation by encapsulating antigen in PLGA particles. Particles of mean size < 500 nm were better inducers of CTL than larger microparticles (mean > 2 microns and above) (164). There are reports indicating that exogenous antigens associated with small particles were presented 1000- to 10,000-fold more efficiently in MHC class I than soluble antigen (165, 166).

Microencapsulated antigens taken up by macrophages have been shown to be displayed on both MHC class I and class II molecules and presented to T cells *in vitro*. The efficiency of antigen presentation correlated well with the phagocytosis of the particles (167). A recent study has demonstrated that particulate antigen size influenced their intracellular localizations and modulated the efficiency of antigen presentation. The phagocytosed particulate Ag that was 560 nm in diameter, but not 155 nm, had the ability to enter early phagosomes and resulted in more efficient Ag presentation (168). Taken together, it would appear the efficient induction of immune responses could be achieved by using PLGA particles of appropriate size and hydrophobicity for antigen delivery.

1.7.7 Antigen release profile

Most vaccine immunogens incorporated into the PLGA nanoparticles were released in a sustained-release manner based on the data from *in vitro* studies (142, 169, 170). The release of antigen from PLGA particles is principally mediated by two mechanisms. One involves diffusion from the surface and through tortuous, water-filled micro-porous channels in the polymer matrix, another one via polymer swelling and bulk erosion polymer degradation (171). Both release mechanisms can occur concurrently (172). The immune response generated from antigen-loaded PLGA particles was in accordance with the *in vitro* release profile of the antigen (162), which is mainly affected by the porosity and the degradation rate of the particles. The porosity of the polymeric particles has been shown to accelerate the diffusional release of antigen (173, 174), resulting in a high initial burst release and an overall augmentation of release rate (155). PLGA particle degradation rate, which is influenced by the polymer molecular weight, lactic to glycolic acid ratio and surface area (175), correlates well with the antigenic protein release (159). Although there is no systematic study to compare the vaccine release kinetics which would produce an optimal immune response to a given antigen, it has been suggested the vaccine release pattern should mimic the antigen concentration seen over time during natural infections (176). In other words, it will be ideal to have a relatively high antigen dose available to the immune system initially. This is followed by a steady decrease of the antigen dose in the body over time. An initial high antigen dose exposed to the immune system has been shown to affect the extent of memory T cell formation (68). The subsequent steady decrease in antigen load may also enhance the development of antibody affinity maturation and the maintenance of high antibody titres (154).

1.7.8 Importance of adjuvants in the vaccine formulations

Immunological adjuvant has been defined as substances, which when used in combination with a specific antigen enhance the immune response to the antigen (177). Most human and veterinary vaccines are commonly adjuvanted with precipitated aluminium salts or alum. Alum-based vaccine formulations produce a higher and longer-lasing primary immune response compared to immunization with soluble antigen alone (178). A new adjuvant that is licensed for human use MF-59 is an oil-in-water submicron emulsion containing squalene. Extensive preclinical data clearly demonstrated the immune potentiating effects of MF-59. Immunization with MF-59-adjuvanted influenza vaccine induced haemaglutinin-specific antibody titers significantly higher than those induced by the non-adjuvanted vaccine (179-181). The strong adjuvant effect of MF59 was also demonstrated in the hepatitis B prophylactic vaccine studies. MF59 in association with a recombinant hepatitis B virus (HBV) vaccine, PreS2+SAg, was found to induce much higher anti-HBs titers than those from alum-adjuvanted vaccine in both primates and humans (182). However, vaccines formulated with MF-59 result in antigen-specific IgG antibodies in serum and Th2 cytokine secretion profile, predominantly a Th2 type of immune response (183, 184). Thus, MF-59, like aluminum salts, preferentially polarizes T cell response towards a Th2 phenotype, and is not suitable to be used as an adjuvant for hepatitis B virus therapeutic vaccine designed to elicit protective Th1-type immune responses.

1.7.9 Monophosphoryl lipid A

1.7.9.1 Immunopotentiating effects

One promising adjuvant candidate is monophosphoryl lipid A (MPLA), which is known to induce potent Th1 immune responses to co-administered antigen with a well-established safety and tolerability profile in humans (185, 186). MPLA is a chemically modified lipid, derived from the lipid A region of lipopolysaccharide from *Salmonella minnesota.* It retains the immune stimulatory properties of LPS, but is devoid of apparent toxicity or pyrogenicity. MPLA is capable of inducing the production of pro-inflammatory cytokines from macrophages (187) and IL-2 and IFN-γ from lymphocytes (188). It has emerged as an effective adjuvant for the induction of both humoral and cell-mediated immune responses (189, 190).

Induction of dendritic cell maturation is critical for activation of naïve T cells. MPLA has been shown to promote dendritic cell activation and maturation by up-regulating costimulatory molecules (i.e., CD80 and CD86), MHC class II and CD40 on dendritic cells (191-193). In addition, MPLA increases the antigen presenting activity of dendritic cells by triggering their migration to the lymphoid organ (194). The number of antigen-pulsed dendritic cells reaching the lymph node has been implicated in the magnitude and quality of induced CD4$^+$ T cell responses (195). Efficient mobilization of DC to the lymphoid tissue may facilitate DC/T cell interactions and the induction of T-cell responses.

It is now known that the innate immune activation by MPLA is mediated by toll-like receptors (TLRs) (196). TLRs function as pattern recognition receptors (PRRs) in mammals. They recognize conserved pathogen-associated molecular patterns (PAMPs) on various microbial components. TLR activation and signaling stimulate innate immune cells, which is important in triggering acute inflammatory responses and the initiation of the adaptive immune response (197). Engagements of TLR2 and TLR4 by MPLA were shown to be involved in the up-regulation of CD80 and CD86 expression and the production of TNF-α, IL-10, and IL-12 by human monocytes (196).

1.7.9.2 Modulation of immune response

Distinct TLR ligand engagement on dendritic cells can determine the type of Th cell responses (198). Via signaling through TLR4, LPS induced a predominant Th1 response by stimulating IL-12 production by dentritic cells (198, 199). Production of IL-12 p40 was observed following MPLA stimulation of human dendritic cells (192) and macrophages (193). Similar to the immunomodulatory effect of LPS, several studies reported that MPLA stimulated an antibody response of IgG_{2a} isotype (200) and preferentially induced a Th1-response (193, 201-203). Elimination of HBV during antiviral therapy correlated with the activation of T cell responses and the Th1-cytokine patterns secreted by T cells in the liver (121). The ability to control the differential induction of Th1 and Th2 responses may hold the key to a successful immunotherapy by altering the clinical course and disease process of chronic hepatitis B.

TLRs play an essential role in the control of the adaptive immune response (197, 204). A recent report suggested that TLR recognition of microbial products such as LPS is critical to block the suppressive effects of $CD4^+CD25^+$ regulatory T cells (T_{reg}), which allows activation of pathogen-specific adaptive immune responses (205). Hence, the inclusion of TLR-agonist based adjuvant (i.e. MPLA) in the vaccine preparation may overcome the $CD4^+CD25^+$ T cell-mediated immune suppression (206), and allow effective stimulation of HBV-specific immune responses and ultimately lead to the resolution of chronic infection.

1.8. Animal models for the study of hepatitis B

Several animal models are available for the study of hepatitis B including mice, chimpanzees, woodchucks and Pekin ducks. HBV transgenic mice have been developed to investigate the pathogenesis of HBV. However, since the hepatitis B viral genes were integrated into the genome of the transgenic mice, the viral antigens were generally considered as self, and tolerated by the immune system of the transgenic mice. Moreover, the HBV in the transgenic mouse does not form an episomal cccDNA, which is the template for viral replication during HBV infection. Elimination of cccDNA is a critical parameter for measuring the resolution of infection. A complete elimination of HBV cannot be assessed in this model. Chimpanzee is susceptible to HBV although it is not a natural host for HBV. The major downside of using this animal model is the cost. In addition, they are generally not accessible for many researchers.

Woodchuck hepatitis B virus model and duck hepatitis B virus model have been used for studying the viral replication, transmission and antiviral therapy. Although the immune systems of woodchuck and Pekin duck have not been well characterized, they are useful preclinical animal animals of HBV infection.

1.9 Duck immune system

1.9.1 Duck antigen presenting cells

Duck hepatitis B virus (DHBV) infected-Pekin ducks have proven to be a useful animal model for the study of hepatitis B virus. A close relationship between immune reactivity to the virus and the outcome of infection has also been observed in DHBV infected-Pekin ducks (64, 207). However, the molecular/biochemical basis of the cellular immune system in ducks has not been well characterized. Avian immune function is presumed to be similar to that of the mammalian species. There is evidence that T lymphocytes of ducks were functionally similar to mammalian and chicken T lymphocytes (208). Co-culturing duck APC with duck lymphocytes has been shown to optimize *in vitro* proliferative responses to low doses of phytohemagglutinin (PHA) (1 μg/mL) and concanavalin A (Con A) (5 μg/mL) (104, 209). *In vitro* proliferative responses of chicken peripheral blood leukocytes to pokeweed mitogen were adherent cell dependent. The adherent cell population contained more than 90% macrophages after 72 h of culture from the peripheral blood mononuclear cell (PBMC) preparation (210). Moreover, T cell proliferation to antigen- pulsed peripheral blood adherent cells (presumably APC) in chicken was MHC class II-restricted (211). Most published reports of duck macrophage cultures were prepared by culturing plastic-adherent PBMC, after isolation from Ficoll-Paque™ density gradients (212-214). It will be useful to determine whether duck macrophages are capable of phagocytosing PLGA nanoparticles and the factors that govern their cellular uptake. This will allow us to investigate the effectiveness of PLGA nanoparticles as a vaccine delivery system using the duck model for hepatitis B infection.

1.9.2 A genomic approach to duck immune gene discovery

The expressed sequence tag (EST) approach is designed to identify large numbers of expressed genes to search for genes critical for immune function in ducks. We have focused on two genes, MHC class II and invariant chain, to allow us to identity antigen presenting cells in the duck. MHC is a large genetic region of genes, which controls and regulates the immune response. DR-A sequences are highly conserved among different species. Chicken is a close relative of duck. The nucleotide sequence, putative amino acid sequence and secondary structure of chicken MHC class II (B-LA) are similar to the HLA-DRA in humans and I-Ea in mice (215, 216). MHC class II –associated invariant chain of chicken also shares many functional properties with its mammalian homologs (217). Because MHC class II molecules and invariant chain are generally expressed in professional antigen presenting cells only, the identification and characterization of their homologues in ducks may help in differentiating APC from other blood cells.

1.10 Duck hepatitis B virus model

Duck hepatitis B virus (DHBV) is closely related to human hepatitis B virus (6). The DHBV genome contains three overlapping open reading frames (ORFs) instead of four as found in HBV (reviewed by Jilbert (207)). The C-ORF encodes the precore and core protein, the S-ORF encodes the envelope protein and the P-ORF codes for the viral polymerase. The core protein forming the inner nucleocapsid of the virion is important for binding to the viral pregenomic RNA for encapsidation during the viral replication process. Some of the mature nucleocapsid containing the viral DNA and polymerase are transported to the nucleus to increase the pool of cccDNA molecules within the infected hepatocytes while some of them are enveloped by the surface protein (DHBsAg) to form a complete virion and exported from the cells.

Like other members of the *Hepadnaviridae* family, DHBV causes either transient or persistent infection in its natural host Pekin duck (*Anas platyrhynchos*). The outcome of the infection is largely dependent on the age of the host at the time of exposure and the dose of virus inoculum. Persistent infection resulted when the ducklings were infected early after hatching. In contrast, infection of older ducks (older than 8 days) usually resulted in transient infection with the development of neutralizing antibodies (218). A minimal and non-significant cellular immune response to both DHBsAg and DHBcAg viral antigens was found in DHBV chronically infected ducks (219). The establishment of chronic infection was associated with the ineffectiveness of the duck immune response to eliminate DHBV. The immune hyporesponsiveness to viral antigens during chronic DHBV was also analogous to human chronic HBV infection. These provide the basis for evaluating the efficiency of vaccine therapy in DHBV infected ducks to induce effective antiviral responses to facilitate the elimination of the virus.

1.11 Research project

The goal of this research is to evaluate PLGA nanoparticles as an antigen and adjuvant delivery system for the induction of cellular immune responses. The physiochemical properties of HBcAg-loaded PLGA nanoparticles were characterized and the immune-enhancing effects of co-delivery of hepatitis B core antigen and monophosphoryl lipid A were investigated in mice. To determine whether the immune responses elicited by the PLGA nanoparticle delivered vaccine could intervene in a natural viral infection, the Pekin duck (*Anas platyrhynchos*) infected with duck hepatitis B virus was used. Duck MHC class II DR-A and invariant chains genes have been cloned and characterized with the express purpose of allowing identification of antigen presenting cells. Antigen uptake by professional antigen presenting cells is an important step for the induction of cellular immune response. The size of PLGA particles and the kinetics of the their cellular uptake by duck macrophages were evaluated *in vitro*.

1.11.1 Hypotheses

1) Co-administration of vaccine antigen and adjuvant in the form of PLGA nanoparticles enhances cellular immune responses and favours Th1 development.
2) Co-delivery of HBcAg and MPLA in PLGA nanoparticles is capable of enhancing HBcAg-specific Th1 immune response and promoting IFN-γ production.
3) PLGA nanoparticles are taken up duck antigen presenting cells.
4) Duck antigen presenting cells express MHC class II-DR like molecules and invariant chains.
5) PLGA nanoparticle formulation containing both DHBcAg and MPLA is capable of generating effective antiviral immune responses in ducks to facilitate viral clearance.

1.11.2 Objectives

1) To examine whether viral antigen-specific Th1 responses can be induced by appropriate vaccine formulation, which includes administering vaccine antigen accompanied by adjuvant in a vaccine delivery system.
2) To investigate the factors associated with particle uptake by duck APC.
3) To characterize a MHC class II-DR like molecule and invariant chains in Pekin ducks, markers of duck APC
4) To examine the duck immune response to duck hepatitis B core antigen – loaded in PLGA nanoparticles.
5) To monitor viremia in chronically DHBV-infected ducks following the immunizations with PLGA nanoparticle formulation.

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Chapter 2

Enhancement of T Helper Type 1 Immune Response Against Hepatitis B Core Antigen by Poly(D,L-lactic-co-glycolic acid) Nanoparticle Vaccine Delivery

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2.1 Introduction

Hepatitis B infection remains a global problem despite the effectiveness of the hepatitis B virus (HBV) vaccine in preventing infection. Approximately 400 million people around the world are persistently infected by HBV (1). These individuals harbouring the virus may continue to transmit the virus and spread the disease. Of greater concern is that majority of these patients may develop cirrhosis or hepatocellular carcinoma later in life as a result of chronic hepatitis (2). Among the adult patients who were infected with HBV, about 95% experienced acute infection with self-limited disease, and 5% developed chronic disease with persistent viremia and liver inflammation. This is in stark contrast to the neonatally-acquired HBV, where ~95% of the infected infants resulted in chronic infection (3, 4).

Although the factors that contribute to the clearance of the virus in the acute self-limited HBV infection or the persistence of the virus in chronic infection are not completely clear, it appears that the disease activity correlates closely with the strength of virus-specific T-cell immune responses. A weak, undetectable and narrowly-focused HBV-specific T cell response is associated with chronically infected patients, whereas strong, polyclonal and multispecific T cells are associated with self-limited acute infection (5-7). A previous study has shown that the vigour of virus-specific immune responses is restored during the spontaneous recovery of chronic infection (8). Correspondingly, the recovery of virus-specific immune responses coincide with a reduction of viral load during lamuvidine treatment (9).

Of various antigens, the viral nucleocapsid hepatitis B virus core antigen (HBcAg) seems to be recognized most efficiently by HLA class II-restricted immune response in acutely infected patients (10, 11). It has been suggested that HBcAg-specific Th cells may also provide "intermolecular T-cell help" to HBsAg-specific B cells and stimulate the production of neutralizing antibody against re-infection (12). Strong HBcAg-specific CD4$^+$ T cell responses are associated with hepatitis B surface antigen (HBsAg) seroconversion, high

$HBcAg_{18-27}$-specific CTL precursor frequency, and control of viremia during acute infection or during IFN-α treatment of chronic HBV patients (13, 14). Adoptive transfer of hepatitis B core antigen-reactive T cells is associated with resolution of chronic HBV infection (15). Clearly, HBcAg-specific $CD4^+$ T cell responses play a central role in the control of HBV (16). Furthermore, a Th1 cytokine profile of core-specific T cells was found predominantly in patients who had successful cleared the virus (17). Based on these strong immune-related evidences for HBV clearance, a vaccine designed to generate robust viral core-specific immune responses, specifically of Th1 type, may provide the key to the successful treatment for chronic HBV infection.

Poly(D,L-lactic-*co*-glycolic acid) (PLGA) polymer is both biocompatible and biodegradable. It has been used as suture material with a well-established safety profile in humans. Controlled release drug delivery systems using this biomaterial has been approved for human use (18, 19). Several studies have shown that PLGA micro and nanoparticles can be used to modulate the immune responses against encapsulated antigens due to their ability to efficiently target professional antigen presenting cells (APC), and facilitate appropriate processing and presenting antigens to T cells (20-25). Monophospholipid A (MPLA), a non-toxic analog of Lipid A has been used in vaccines to enhance Th1 and CTL responses in animals and humans (26-28). Co-delivery of hepatitis B antigen with MPLA allows simultaneous targeting of the antigen and the Th1 promoting adjuvant, i.e., MPLA, to the same APC by PLGA nanoparticles. This represents a logical strategy to activate protective immune responses and improve the efficacy of the immunotherapeutic vaccines for chronic HBV. The protein stability and the antibody responses has been examined for rHBcAg-loaded PLGA microspheres (29). In this study, the physiochemical properties of HBcAg-loaded PLGA nanoparticles were characterized further and the immune-enhancing effects of co-delivery of antigen and MPLA in mice were investigated.

2.2 Materials and Methods

2.2.1 Nanoparticles preparation

Poly(D,L-lactic-*co*-glycolic acid) (PLGA) polymer with inherent viscosity of 0.16 dL/g and an average of 6000 g/mol molecular weight was purchased from Birmingham Polymers, Inc., Birmingham, AL. Recombinant HBcAg (adw) with a c-terminal His-tag (156 amino acid: MDIDPYKEFGASVELLSFLPSDFFPSIRDL LDTASALYREALESPEHCSPHHTALRQAILCWGELMNLATWVGSNLEDPASRE LVVSYVNVNMGLKIRQLLWFHISCLTFGRETVLEYLVSFGVWIRTPPAYRPPNA PILSTLPETTVVRHHHHHH), produced in E. *coli*, was obtained from Chemicon International, Inc., Temecula, CA). It was tested and shown to be endotoxin-free (<0.01 EU/μg of rHBcAg). Poly(vinyl alcohol) (PVA) (molecular weight 31,000-50,000 g/mol, 89% hydrolyzed) was obtained from Sigma-Aldrich Canada, Ltd., Oakville, ON, Canada. Chloroform (HPLC grade) was purchased from Fisher Scientific International, Inc., Nepean, ON, Canada. Synthetic MPLA was kindly donated by Biomira, Inc., Edmonton, AB, Canada (30-32). Three different nanoparticulate formulations: (HBcAg + MPLA) in PLGA nanoparticles; (HBcAg) in PLGA nanoparticles; (MPLA) in PLGA nanoparticles; were made by a water/oil/water (w/o/w) solvent evaporation method (33). Briefly, 20 μg of HBcAg (0.83 mg/mL w/v; 24 μL) was emulsified in a solution of PLGA in chloroform (100 mg in 400 μL) by sonication at level 4 for 15 second using a microtip sonicator. (Model XL 2010; Heat Systems, Inc., Farmingdale, NY). This primary emulsion was then further emulsified in 2 mL 9% w/v polyvinyl alcohol (PVA) in phosphate buffered solution (PBS) with sonication at level 4 for 20 second to form a secondary emulsion. The resulting double emulsion was then added drop-wise to a stirring solution of PVA/PBS (9% (w/v); 8 mL). The emulsion was left to stir at room temperature for 3 hours to allow the evaporation of chloroform. The resulting nanoparticles were washed three times with sterile water (4 °C) and frozen immediately in a dry ice/acetone bath. They were then freeze-dried under vacuum for 3 days. Sterile PBS was used instead of the rHBcAg antigen in the primary emulsion formation to prepare the nanoparticles without the rHBcAg. For the nanoparticle preparation containing MPLA (e.g., (MPLA) nanoparticles and (HBcAg + MPLA) nanoparticles), 100 μL of MPLA solution (2 mg/mL (w/v) in chloroform/methanol mixture (4:1 (v/v)) was added to the polymer solution before the formation of the primary emulsion.

2.2.2 Estimation of protein loading in nanoparticles

Micro-BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL) was used to determine the rHBcAg loading in the nanoparticle formulations. Extraction of protein from the particles was prepared by stirring the particle samples (40 mg) in 5% sodium dodecyl sulphate (SDS)/0.1 M NaOH w/v overnight (34, 35). The sample was then neutralized to pH 7 with 1 M HCl. Water was added to bring the final volume to 1 mL before analysis. Micro BCA assay was performed in a 96-microwell plate (Gibco BRL) to determine protein concentration. A fresh set of protein standards was prepared by diluting the 2.0 mg/mL BSA stock standard (linear working range of 1-20 μg/mL, $r^2=0.9753$). The absorbance of the samples was measured at 562 nm by a scanning spectrophotometer (PowerWave X; Bio-Tek Instruments, Inc., Winooski, VT). To determine whether the presence of MPLA may interfere with the Micro-BCA assay, 12 μg of MPLA (1 mg/mL) per well was added to the (HBcAg) nanoparticles collected samples (150 μl/well) to compare the protein quantification results from the (HBcAg) nanoparticles in the absence of MPLA.

2.2.3 Analysis of MPLA content in nanoparticles

MPLA content was analyzed by HPLC according to the procedure reported by Hagen et al. (36) with some modification. Briefly, MPLA was derivatized by reacting with a solution of dinitrobenzyloxyamine hydrochloride (Sigma-Aldrich) in anhydrous pyridine (10mg/mL; 200 μl for an estimate of 1 mg MPLA extracted from nanoparticles). The samples were dried under N_2 gas at 60 °C and then dissolved in 500 μl $CHCl_3$-CH_3OH (2:1, v/v). The HPLC assay was carried out with μBondapak™ C_{18}, 10 μm particle size, 3.9 x 300 mm stainless steel column (Waters Corp., Milford, MA). Data were collected and processed with an Allchrom™ Plus data system (Alltech Associates Inc., Deerfield, IL). Two mobile phases consisted of (A) 5mM tetrabutylammonium dihydrogenphosphate (TBAP) (Sigma) in acetonitrile-water (95:5, v/v) and (B) 5mM TBAP in isopropanol-water (95:5, v/v), was delivered at 1.0 mL/min by increasing the gradient from 10% to 80% B in 15 min and restoring to 10% B over 30 min. The samples were detected at 254 nm with an UV-detector. The calibration curve used for the quantification of MPLA in the nanoparticles was linear over the range of 50 μg- 250 μg with a correlation coefficient of r^2 = 0.9969. The reagents used in the experiments were of HPLC grade.

2.2.4 Surface morphology of nanoparticles

To examine the surface appearance of the nanoparticles, the PLGA nanoparticles were dispersed on a metal stub and allowed to dry. The samples were then placed in a sputter coater (S150B; BOC Edwards, Sussex, UK) for 40 s to produce a gold coating of approximately 30 nm in thickness. They were viewed under a scanning electron microscope (Hitachi S-2500; Hitachi, Ltd., Tokyo, Japan).

2.2.5 Measurement of particle sizes

The mean size diameter of the nanoparticles was determined by photon correlation spectroscopy (PCS) (Zetasizer 1000HS; Malvern Instruments, Ltd., Cirencester, UK) at 633 nm. Samples of PLGA nanoparticles dispersion (3 mL; 0.07 μg/mL w/v) in 1 mM of NaCl were placed in a cuvette for size measurements. CONTIN analysis was performed to measure the size and distribution of the particles.

2.2.6 *In vitro* release study of ^{125}I-radiolabeled HBcAg-loaded nanoparticles

Hepatitis B core protein was labelled with iodine radioisotope as described previously (37). 5 μL of IODO-GEN® (Pierce Biotechnology, Inc., Rockford, IL) in chloroform (0.005% v/v) was plated in a glass test tube. Twenty microliters of ^{125}I-NaI (specific activity: 100 mCi/mL; Nycomed Amersham Canada, Ltd., Oakville, ON. Canada) and 10 μL of rHBcAg (2.93 mg/mL) were added in sequence. The reaction was allowed to proceed for 45 minutes at room temperature. The radiolabelled rHBcAg was separated from the free ^{125}I-NaI by Sephadex® G-25M size-exclusion column (Pharmacia Corp., Peapack. NJ). The radioactivity was measured in 1480 Wizard 3 automatic gamma counter (Wallac, Turku, Finland). The protein fraction used in the study contained 98% of radioiodine in a protein bound form, determined by the TCA precipitation method. ^{125}I-radiolabelled rHBcAg was then formulated in PLGA nanoparticles in the presence or absence of MPLA by the method described earlier. PLGA nanoparticles (450 mg) containing rHBcAg (~10 μg) were suspended in 30 mL PBS (pH 7.32) containing 0.02% v/v Tween 20 and 0.01% w/v sodium azide. Thirty microcentrifuge tubes containing 1 mL aliquot of the suspended particles (15 mg) was placed in a 37°C water-bath and shaken at level 3. At designated time intervals, a set of triplicate samples was removed and the supernatant was separated from the particles by centrifugation. TCA precipitation was used to measure the protein bound radioactivity released in the supernatant. The percentage of ^{125}I-labeled HBcAg released from the nanoparticles was calculated based on the protein bound radioactivity. The results are shown in Figure 2-2.

2.2.7 Immunizations

Pathogen-free female C57BL/6J mice were obtained at 8-10 wks of age from Charles River Laboratory (Wilmington, MA). The care and the use of laboratory animals complied with the Canadian Council on Animal Care (CCAC) guidelines. There were 9 groups of mice in this study. Five groups of mice received a single immunization regimen. The other 4 groups of mice were vaccinated twice with the same vaccine formulations, and they are designated as booster-dose groups. On day 1, each group of mice (n=5) was immunized subcutaneously with one of the following formulations: (MPLA) PLGA nanoparticles; (HBcAg) PLGA nanoparticles; (HBcAg + MPLA) PLGA nanoparticles; soluble HBcAg; HBcAg and MPLA mixture in 200 μL PBS. The mice in the single immunization groups were sacrificed on 9 days after the immunization. The other groups of mice who received a booster dose on day 14 were euthanized later on day 21. Blood samples were collected through cardiac puncture and the spleens and the inguinal lymph nodes were isolated for the immunological assays.

2.2.8 T cell proliferative assay

T cells were isolated from inguinal lymph nodes and spleens of the euthanized mice. Briefly, a single-cell suspension was prepared by smearing cell aggregates between two frosted slides. Nylon wool column, consisting 5 g of nylon wool (type 200 L; Robbins Scientific Corp., Sunnyvale, CA) in a 10 mL BD-syringe was used to enrich T cells from the cell suspensions. T cells (5×10^5) were incubated with irradiated (3000 rad) autologous spleen cells (1×10^6) with appropriate peptides (20 μg/ 200 μL media per well) and protein as recall antigens (1 μg /200 μL media per well) at 37 °C and 5% CO_2 or an irrelevant protein hen egg lysozyme (HEL; Sigma-Aldrich Chemical Corp.). The H-2^b Th-epitope, HBcAg peptide fragment (129-140: H_2N-PPAYRPPNAPIL-C00H) was synthesized on a solid-phase synthesizer with >95% purity as determined by HPLC (Beckman System Gold HPLC system). After 72 hours, 1 μCi ^3H-thymidine (1mCi/mL Amersham, Oakville, ON, Canada) in 50 μL of RPMI-10 was added to each well. Twenty-four hours later, the cells were harvested on to a filtermat A (Wallac, Turku, Finland.) using a cell harvester (Mach III Harvester 96®; Tomtec, Inc., Orange, Conn). A melt-on scintillator (1450-441 MeltiLex™ A) was used to measure incorporated radioactivity in a liquid scintillation counter (1450 Microbeta Wallac Trilux). All samples were prepared in triplicate. Results are presented as stimulation index, which is the ratio of the radioactivity of the cell culture in the presence of antigen to that obtained without antigen. A stimulation index of ≥ 3 was considered significant (38).

2.2.9 Antibodies assays

Serum samples obtained from the mice were analysed for the HBcAg-specific IgM and IgG antibodies responses. Normal serum obtained from naïve mice was used as a control. A 96-well microtiter plate (Nunc-immunoplate Maxisorp, Nunc, Roskilde, Denmark) was coated with 100 ng of rHBcAg in 200 μL of PBS per well overnight at 4°C. The plates were washed three times with PBS. The wells were blocked with a 1% (v/v) BSA solution (Kirkegarrd & Perry Laboratories, Inc., Gaithersbury, MD) at 37°C/5% CO_2 for 30 minutes. The plates were again washed three times with TPBS (0.05% v/v Tween 20 in PBS) and tapped dried. A serial dilution of the tested sera was added to the wells and allowed incubation at room temperature for 1 hour. The wells were then washed five times with TPBS, and incubated with 100 μl of peroxidase-labelled goat anti-mouse IgG or IgM (0.5 μg/mL; Kirkegarrd & Perry Laboratories, Inc., Gaithersbury, MD) for 1 hour. Unbound antibodies were removed as the plates were washed six times with TPBS. A 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) substrate was added for colour development. A 15-minute OD reading at 405 nm was measured using a microplate scanning spectrophotometer (PowerWave X; Bio-Tek Instruments, Inc., Winooski, VT). Data were expressed as the mean OD value of the samples minus the mean OD value of the control. The data were analysed using the KC junior Win software program (Bio-Tek Instruments, Inc., Winooski, VT).

2.2.10 Cytokine assays

Supernatants collected from 72-hour culture in the T-cell proliferation assays were kept frozen at -22°C until analysis. The cytokines IFN-γ and IL-4 were quantified by a sandwich enzyme immunoassay. Microtiter plates (96-well) were coated with the 1st antibodies in 50 μL/well. The plates were coated with the 11B11, antibody for IL-4 (Biomira, Inc., Edmonton, AB, Canada) at 25 ng/well or 75 ng/well of R46.A2 antibody for IFN-γ (Biomira, Inc., Edmonton, AB, Canada) for 30 minutes at 37°C and 5%CO_2. The plates were then washed once with TPBS. Sets of standard serial dilution of rIFN-γ (2 ng/mL w/v) and rIL-4 (0.5 μg/mL w/v; BD-Pharmingen Canada, Inc., Mississauga, ON, Canada) were prepared and added to the corresponding plates along with the test supernatants. For IFN-γ, the standard rIFN-γ was diluted with the supplemented RPMI-1640 media to a series of concentration ranging from 5000 to 156 pg/mL.

In the case of rIL-4, the standard rIL-4 was prepared as a series of concentration from 1666 to 14 pg/mL. After 45-minute incubation at 37 °C, the wells were washed two times with TPBS. The second antibodies were added to the plate in 50 μL/well. XMG1.2, Biotinylated antibodies for IFN-γ were added the IFN-γ test plate at 2.5 ng/well whereas BVD6-24G2, biotinylated antibody for detecting IL-4, was added at 10 ng/well, followed by 45 minutes incubation at 37°C. The plates were washed three times with TPBS to remove unreacted antibodies. A solution of peroxidase-conjugated streptavidin (50 μL) solution (Jackson ImmunoResearch Lab, Inc., West Grove, PA) was added at 10 ng/well in 1% w/v BSA/TPBS. After 30 minute of incubation at 37°C, the plates were washed 4X with TPBS. A 100 μl final volume of a solution mixture containing 3,3,5,5-tetramethylbenzidine (TMB) substrate was added to each well according to the manufacturer instruction (KPL). A kinetic reading was measured over 2 minute at 650 nm. An endpoint reading 450 nm was determined by adding 100 μL of 1 M phosphoric acid per well to stop the colour reaction.

2.2.11 Statistical analysis

SAS software program (SAS Institute Inc., Cary. NC. USA) was used to analyse the collected data. The t-test procedure was applied to study the effect of MPLA (12 μg/well) on the micro BCA assay used for protein quantification (α= 0.05). Analysis of covariance (ANCOVA) was used to test for the difference in protein release rates (i.e. heterogeneity of slopes) between the two particulate vaccine formulations (α= 0.05). The data collected from the T-cell proliferation assay were analyzed by a multiple comparison test Student-Newman-Keuls (S-N-K) (α= 0.05). This test is only utilized if the one-way ANOVA test result was found to be significant (α=0.05).

2.3. Results

2.3.1 Particle characterization

The mean volume diameters of (MPLA) nanoparticles, (HBcAg) nanoparticles and (HBcAg + MPLA) nanoparticles were 278 nm (polydispersity: 0.49), 266 nm (polydispersity: 0.53) and 279 nm (polydispersity: 0.17), respectively. The SEM pictures (Figure 2-1) showed that the nanoparticles were spherical, with no cavities and essentially a smooth surface overall in most of the observed particles. No obvious differences were noted with respect to their surface morphologies from the particulate formulations prepared in this study.

Lipids are known to interfere with the Micro BCA protein assay. The presence of MPLA in the amount used for encapsulation in this study did not show any significant effect on protein quantification by the micro-BCA assay ($p=0.4071$). It has been reported that the lipid effects on the Micro BCA assay can be eliminated by the addition of 2% SDS to samples prior to the analysis (39). The presence of SDS in the polymer hydrolytic mixture may explain the minimal lipid effect on the protein assay in this study.

The HBcAg encapsulation efficiency for the (HBcAg) nanoparticles and the (HBcAg + MPLA) nanoparticles were 50.8 % (±0.82) and 51.0 % (±1.76) respectively. The protein mass per dry weight of nanoparticles was equal to 0.0102% w/w for both the (HBcAg) nanoparticles and the (HBcAg + MPLA) nanoparticles. Comparable amounts of core protein HBcAg were loaded in the PLGA nanoparticles irrespective of MPLA incorporation. Approximately 1 μg of core protein was loaded in 10 mg nanoparticles formulations. Thus, a 10 mg of PLGA nanoparticles dose was used for the particulate formulations in the immunization studies. Consistently, each experimental test groups received 1 μg antigen dose per immunization irrespective of their formulations.

The encapsulation efficiency for MPLA in PLGA nanoparticles was 44.95% (±1.954) based on the HPLC analysis. It was estimated that 9 μg of MPLA in form of nanoparticles (10 mg) was given to the animal per immunization. In comparison, the amount of MPLA given in the MPLA + HBcAg vaccine mixture group (20 μg) per immunization was twice as much as those in the particulate formulations. The MPLA used for vaccination was prepared in isotonic saline (1mg/mL) containing 0.2% triethylamine (w/v).

2.3.2 *In vitro* protein release profile

A bi-phasic protein release pattern was displayed in both the ^{125}I-labeled HBcAg protein-loaded PLGA nanoparticles with or without encapsulated MPLA for the studied period (Figure 2-2). Approximately 13% of encapsulated protein was rapidly released in the first 24 hours. A plateau phase with little protein release was observed between 24 to 205 hours. The amount of protein release in the (HBcAg + MPLA) nanoparticles was found to be significantly higher than (HBcAg) nanoparticles ($p<0.0015$), however, a statistical analysis indicated that the protein release rates (i.e. slope) between the two vaccine formulations was not significantly different ($p = 0.7383$).

2.3.3 Antigen-specific immune responses

2.3.3.1 T-Cell proliferative responses with a single immunization

As shown in Figure 2-3 (A-B), the mice immunized with (HBcAg + MPLA) nanoparticles produced the strongest HBcAg-specific T cell proliferation both locally and systemically. Their stimulation indices (SI$_s$) were the highest among all the vaccine groups. SI values against either core peptide (129-140) and core protein reached 17 and 10 in the local lymph nodes and 8 and 5 in the spleen, respectively. Noticeably, when tested against core peptide 129-140, the SI in the (HBcAg + MPLA) nanoparticles immunized group was significantly higher than all the other vaccine groups (ANOVA; $p < 0.0001$ in the lymph node and $p = 0.0006$ in the spleen). The only SI comparison that did not reach statistically significance was in the splenocyte culture when (HBcAg + MPLA) nanoparticles-primed T cells comparing to the HBcAg nanoparticles-primed T cells (S-N-K test). In any case, their T cells proliferate strongly against the relevant recall antigens whether it is in either peptide i.e. HBcAg (129-140) or in particulate form i.e. HBcAg. Especially, in the lymph nodes, the SI from the (HBcAg + MPLA) nanoparticles was ~4 times higher than the un-encapsulated HBcAg. In contrast, the magnitude of the stimulation indices in the lymph nodes compartment in the HBcAg + MPLA, (HBcAg) nanoparticles and the un-encapsulated HBcAg groups were not significantly different from each other (S-N-K test). In addition, a positive core protein dose and T cell response relationship was observed from the (HBcAg + MPLA) nanoparticles-primed T cells *in vitro*.

2.3.3.2 T-cell proliferative responses with a booster immunization

Four groups of mice were immunized with either 1) (MPLA) nanoparticles, 2) HBcAg, 3) HBcAg + MPLA, and 4) (HBcAg + MPLA) nanoparticles on day 1 and received a booster dose of the same formulation on day 14. T cells were isolated from the inguinal lymph nodes and spleens from these mice on day 21. Following the second immunization, the T-cell proliferation profile appears to be different from the single immunization results (Figure 2-4(A-B)). Mice immunized with the HBcAg + MPLA simple mixture had the highest HBcAg-specific T cells activities among the vaccination groups. Second encounters of the same antigen generated a higher T-cell response in the vaccine groups that had previously shown to be weak inducers in a single priming regimen. Comparatively lower SI was observed with a booster dose of (HBcAg + MPLA) nanoparticles than a single immunization. The higher proliferative background found in the un-stimulated T cells from this group may account for the low stimulation index observed in this study by a booster application. Albeit lower T cell proliferative response with a booster injection, co-delivery of HBcAg and MPLA in PLGA nanoparticles in the vaccine formulation displayed strong T-cell responses to the core antigens consistently in both single and booster immunization protocols.

2.3.3.3 Anti-HBcAg antibody responses

None of immunized groups produced detectable HBcAg-specific IgM and IgG day 9 after a single immunization. After receiving a booster dose, anti-HBcAg IgG was found in sera from mice injected with HBcAg containing vaccines (Figure 2-5). A relatively high level of core protein antibody was observed in mice inoculated with HBcAg alone or with HBcAg + MPLA mixture. A lower antibody level was produced in (HBcAg + MPLA) nanoparticles immunized group. Anti-HBcAg IgM remained low in sera after a booster vaccination (Figure 2-6).

2.3.3.4 Cytokine Profile

The cytokine profile of the T cells can be used to distinguish the Th1/Th2 type of immune response. The supernatants from the T cells stimulated with recall antigens HBc peptide (129-140) and HBcAg protein were collected to quantify the production of IL-4 and IFN-γ in the culture. From the single immunization regimen, no IL-4 production was found in all the groups tested (data not shown). Only the HBcAg nanoparticles and (HBcAg + MPLA) nanoparticles immunized groups produce detectable amount of core-specific IFN-γ (Table 2-1). Undetectable IFN-γ was found when the mice were vaccinated with HBcAg alone or HBcAg + MPLA mixture. In contrast, a significant amount of HBcAg-specific IFN-γ was present from the T cell cultures collected from the lymph nodes and spleens of mice that had been immunized with the (HBcAg + MPLA) nanoparticles. In the groups receiving the booster regimen, the relative levels of the core-specific IFN-γ secretions were apparently much higher than the single immunization groups. Particularly in mice that were immunized with (HBcAg + MPLA) nanoparticles, the IFN-γ level had increased 4 and 6-fold in the *in vitro* T cell culture from the spleen and lymph nodes compartment, respectively (Table 2-1). Likewise, no IL-4 was detected from any of the vaccine formulation in the booster groups.

2.4 Discussion

Surface characteristics of the particles can affect the release pattern of protein from polymeric matrix. A porous structure promotes protein release by diffusion, whereas a smooth surface minimizes the burst release (40). PLGA nanoparticles used in this study possessed a cavity-free surface, released 11.64% ± 0.179 of protein from the (HBcAg) nanoparticles and 12.86% ± 0.381 from the (HBcAg+MPLA) nanoparticles into the medium in the first 14 hours. The *in vitro* release pattern of the antigen was previously reported to be in accordance with the appearance and duration of antibodies in the immunized animals (41). This may explain a possible delay in the production of similar or higher level of anti-HBcAg IgG response in mice from the (HBcAg + MPLA) nanoparticles immunized group as compared to the free HBcAg immunized groups.

HBcAg is known to be immunogenic in mice (42). However, at an extremely low dose (1 µg), it failed to produce any detectable antibody or T cell responses after a single subcutaneous immunization. The introduction of MPLA to HBcAg in solution or encapsulation of the HBcAg in PLGA nanoparticles did not result in measurable antigen-specific IgM or IgG humoral responses after a single immunization. However, the addition of MPLA to HBcAg alone was sufficient to elicit cellular immune induction in the local lymph nodes and spleen. It produced a specific-T cell proliferative response (SI>=3) when the primed-T cells were co-cultured with a 12-mer HBc peptide (129-140). Notably, the incorporation of MPLA to the HBcAg-loaded nanoparticles increased the SIs to almost 4-fold compared to the unencapsulated HBcAg. Overall, the T cells isolated from the antigen-encapsulated groups, (HBcAg) nanoparticles and (HBcAg + MPLA) nanoparticles, had significantly higher SIs than the unencapsulated HBcAg vaccinated group (S-N-K).

A general increased in T cell proliferative response was observed from all the tested groups after a booster immunization. It appears that after a second immunization, the soluble mixture of HBcAg and MPLA elicited a stronger T cell proliferative response than the particulate formulation. This result suggests that

MPLA is a potent adjuvant effective in stimulating T-cell activation. However, the IFN-γ production in the group receiving the soluble mixture (HBcAg+MPLA) was significantly lower than group receiving the particulate formulation ((HBcAg + MPLA) nanoparticles). Continuous stimulation of dendritic cells via toll-like receptor (TLR) was found to be required for sustained secretion of proinflammatory cytokines (43). Thus, the PLGA nanoparticulate formulation containing MPLA may provide a sustained release or continuous stimulation of TLR to produce a higher amount of IFN-γ seen in this study.

Previous studies have demonstrated the immunostimulatory properties of PLGA particles for various protein antigens (24, 44, 45). The size of the particles is critical for the uptake by the professional antigen presenting cells (APC) (46, 47). Small particles (mean size <500 nm) were found to induce higher immune responses than large particles (mean size >2 μm) (48). In this study, the PLGA polymer was formulated into particles in nanometer range. It is likely that the particulate formulation in this size range facilitates the uptake by dentritic cells and macrophages, thereby enhancing the processing and presentation of the encapsulated HBcAg and the immune effects observed in this study.

Antigen dose, MHC affinity, time of T-cell receptor engagement, and cytokines milieu during T cells differentiation play a crucial role in influencing Th1/Th2 bias in immune responses (49). The nature of the antigen can also influence the type of immune responses induced. Spontaneous production of IL-12, IL-18, and IFN-γ and Th1 polarization were found as a result of repeated particulate Ag challenge (50). The absence of IL-4 and a high level of IFN-γ production from the (HBcAg + MPLA) nanoparticles-primed T cells in this study indicated a predominantly Th1-immune induction. This is consistent with other research findings (51-55), in which particulate delivery of antigen was found to favour the induction of Th1 immune responses.

Immune-enhancing effects of MPLA have been reported for a number of antigens including the HIV soluble protein (gp 120) and tuberculosis subunit vaccine (26, 56). Via its binding with Toll-like receptor 4 (TLR4), MPLA upregulates the expression of co-stimulatory molecules on APCs and induce the secretion of cytokines such as IFN-γ, TNF-α IL-1β and IL-12 that leads to Th1 cellular immune response (57). The results of this study suggest that co-delivery of antigen and MPLA in nanoparticles was essential for the generation of high IFN-γ level by T cells. Although concomitant delivery of MPLA and HBcAg was capable of enhancing the immunogenicity of a soluble formulation of HBcAg (1 μg), as evidenced by the HBcAg-specific T cell proliferation *ex vivo*, IFN-γ production by the proliferating T cells in this group was significantly lower than that observed in the (HBcAg+MPLA) nanoparticle immunized groups. A significantly higher amount of IFN-γ production was produced from the (HBcAg) nanoparticles primed T cells when MPLA was incorporated in the particles. This effect was even greater in the (HBcAg+MPLA) nanoparticles immunized group after a second immunization.

The observed immunostimulatory effects do not appear to be associated with the antigen release rate. A slightly higher amount of protein was released by the (HBcAg+MPLA) nanoparticle but the difference in the antigen release rates between the (MPLA + HBcAg) nanoparticles and (HBcAg) nanoparticles was found to be statistically insignificant. It is generally believed that the antigen depot effect contributes to the adjuvant effects of many antigen delivery vehicles (58). In the present study, it is not known whether the release pattern of the encapsulated MPLA give rise to the observed immune invigorating effect. A preliminary study was conducted to investigate the burst release pattern of MPLA from the PLGA nanoparticle. It was found that >13% of MPLA was released in the first 24 hours. A systematic study should be carried out in the future to determine if the release pattern of MPLA plays a role on its enhanced adjuvant effects when encapsulated in nanoparticles.

Multiple attempts have been made in the past to treat HBV chronically infected patients with immunotherapeutic vaccines. These efforts include such diverse strategies as using multiple injections of HBsAg (Genhevac–B or Recombivax) (59-61), DNA-based immunization involving intramuscular or subcutaneous administration of DNA expressing vectors encoding sequences of the virus protein (62-64), and lipopeptide-based immunotherapeutic vaccines (Theradigm-HBV) (65). The resulting immune responses induced by these vaccines however, were not very effective in clearing the HBV infection in the chronically infected HBV patients. Therefore, strategies to design an appropriate vaccine, which tailors to increase the strength of Th1 virus-specific immunity, are needed to improve the efficacy of immunotherapy. Furthermore, such vaccine therapy should promote IFN-γ generation, which allows non-cytolytic inhibition and clearance of HBV, thus minimizing the potential risk of massive liver injury.

The central role of TLRs in bridging innate and acquired immune responses is well recognized (66-68). Immunological adjuvants such as MPLA and CpG oligonucleotides function by serving as ligands for TLRs. TLR-mediated 'danger signalling' alerts and prepares the adaptive immune system for defense against foreign antigens. TLR ligands induce maturation and activation of DCs as well as secretion of pro-inflammatory cytokines that can block the suppressive effect of $CD4^{+}CD25^{+}$ regulatory T cells (69). Co-delivery of TLR ligands and antigens to DCs may provide the key to overcome the immune tolerance mechanisms in chronic viral infections. PLGA nanoparticles are suitable for targeting a broad number of TLR analogs and antigens to DCs and can reduce the effective adjuvant dose of TLR analogs significantly (70).

This study highlights the significance of co-delivery of HBcAg with MPLA in biodegradable PLGA nanoparticulate formulation for induction of robust Th1 immune responses and their synergistic effect on IFN-γ production. Such responses are critical for the control of viral replication and the elimination of chronic hepatitis B. The IFN-γ levels can be further amplified by a booster application, especially, with the (HBcAg+MPLA) nanoparticles formulation. Co-delivery of antigens and immunomodulators in a single particulate formulation offers a logical strategy to influence both the quality and the quantity of immune responses. This immunotherapeutic strategy deserves further investigation with respect to immune-mediated viral inhibition and disease resolution in a chronic HBV infection setting.

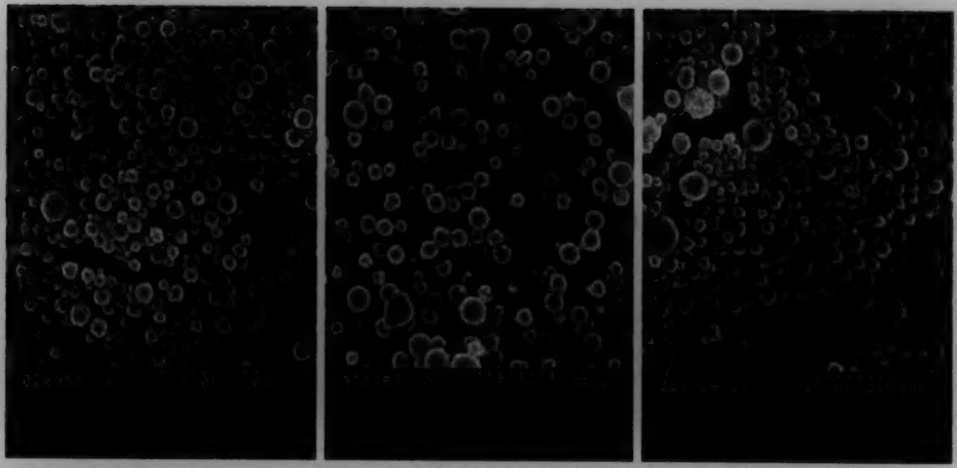


Figure 2-1. Scanning electron micrograph of surface morphology for (MPLA) nanoparticles (left panel), (HBcAg+MPLA) nanoparticles (middle panel) and (HBcAg) nanoparticles (right panel).

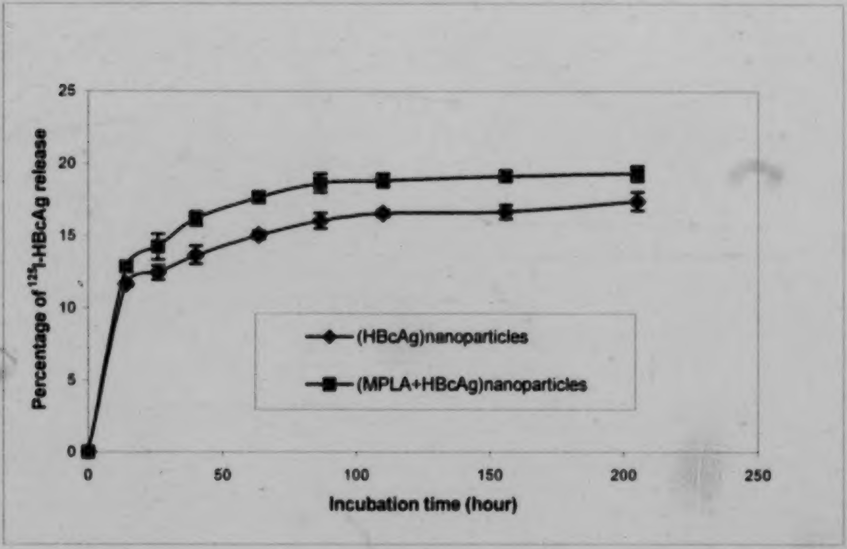


Figure 2-2. Release profile of ^{125}I-labelled HBcAg from PLGA nanoparticles in the presence or absence of MPLA. Samples were incubated at 37°C in phosphate buffer (pH 7.32) containing 0.01% Tween 20. Each point represents the mean ± SD of triplicate samples.

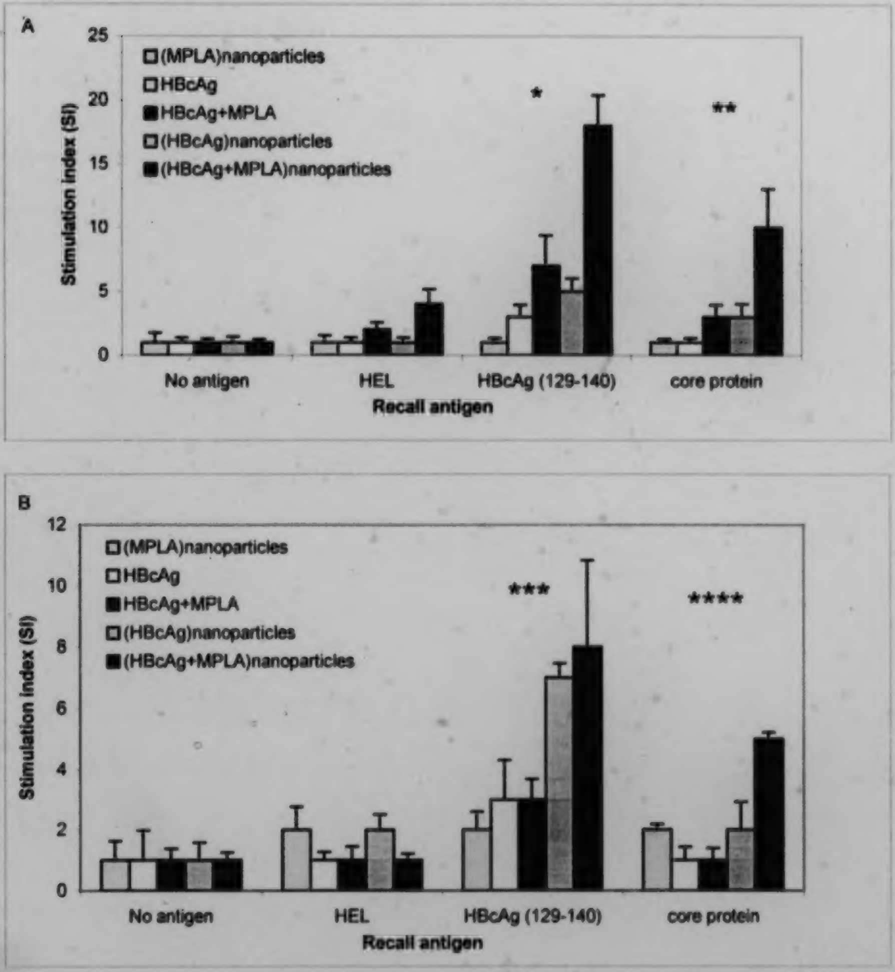
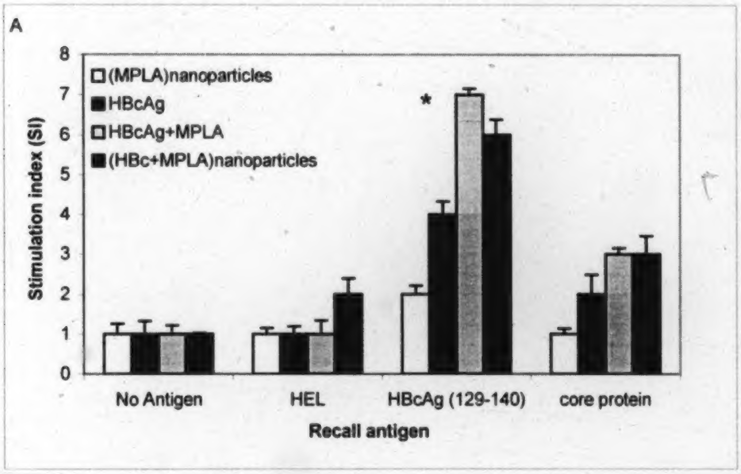


Figure 2-3. T-cell proliferative responses from the single immunization regimen. Isolated T cells from either inguinal lymph nodes (A) or spleens (B) collected from mice that had been inoculated with one of the following formulations: 1) MPLA-loaded nanoparticles, 2) HBcAg alone, 3) HBcAg + MPLA mixture, 4) HBcAg-loaded nanoparticles and 5) (HBcAg + MPLA)-loaded nanoparticles. Stimulation index (SI) was calculated as the ratio between the cpm obtained in the presence of antigen to the background cpm (that obtained without antigen). The cpm values were ranged from 24749 to 43573. ANOVA *$p < 0.0001$; ** $p = 0.0004$; *** $p = 0.0006$; ****$p < 0.0001$



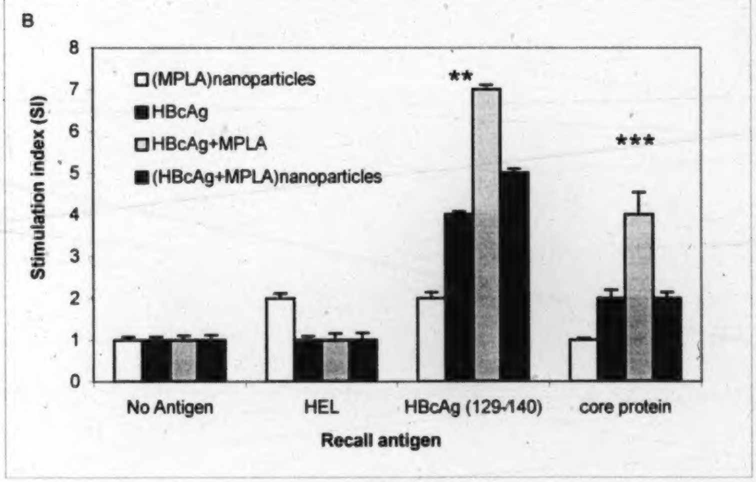


Figure 2-4. T-cell proliferative responses from the booster immunization regimen. Isolated T cells from either inguinal lymph nodes (A) or spleens (B) collected from mice that had been inoculated twice with one of the following formulations: 1) MPLA-loaded nanoparticles, 2) HBcAg alone, 3) HBcAg + MPLA mixture and 4) (HBcAg + MPLA)-loaded nanoparticles. The cpm values were ranged from 15886 to 57746. All samples were prepared in triplicate. ANOVA $^{*}p < 0.0001$; $^{**}p < 0.0001$; $^{***}p = 0.0024$

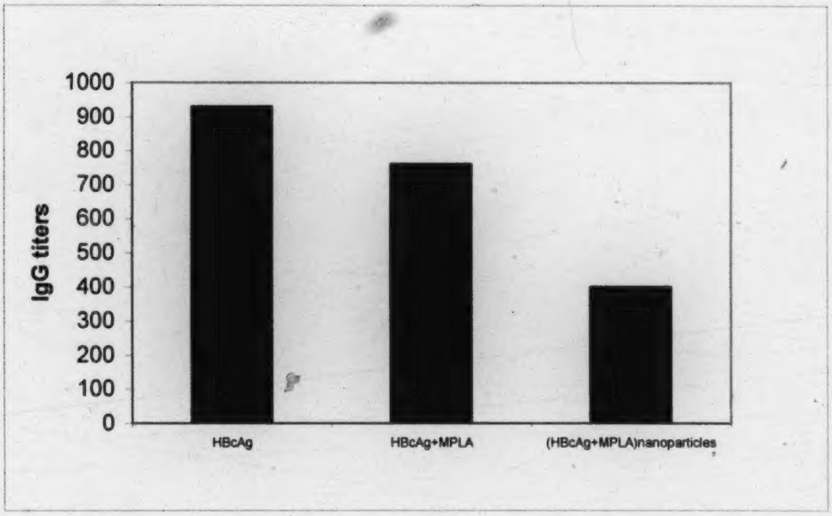


Figure 2-5. Anti-HBcAg humoral immune response (IgG) from the booster immunization groups. Mice were inoculated twice subcutaneously with one the following formulations: 1) HBcAg alone, 2) HBcAg + MPLA mixture and 3) (HBcAg + MPLA)-loaded nanoparticles.

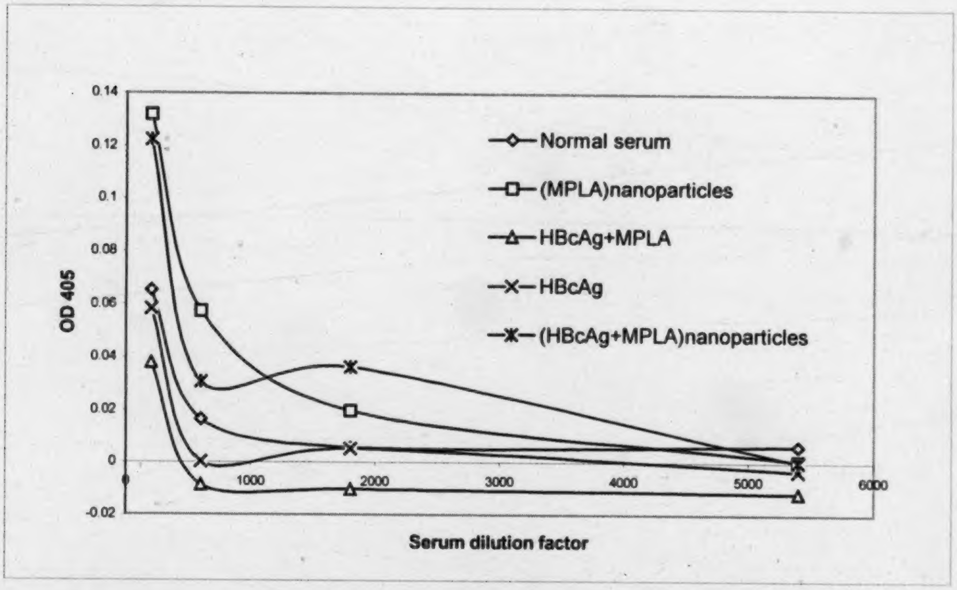


Figure 2-6. Anti-HBcAg humoral immune response (IgM) from the booster immunization groups. Mice were inoculated twice subcutaneously with one the following formulations: 1) MPLA-loaded nanoparticles, 2) HBcAg alone, 3) HBcAg + MPLA mixture and 4) (HBcAg + MPLA)-loaded nanoparticles. The OD 405 absorbance reading represents the serum antibodies reactivity against HBcAg.

IFN-γ assay Average ± SD (pg/mL)		Lymph Nodes		Spleens	
Recall antigen		Single	Booster	Single	Booster
HBc peptide	(MPLA) nanoparticles	UD	UD	UD	308 ± 24
(129-140)	HBcAg	UD	214 ± 106	UD	1195 ± 63
	HBcAg + MPLA	UD	344 ± 56	UD	1234 ± 88
	(HBcAg) nanoparticles	188 ± 21	NA	465 ± 68	NA
	(HBcAg+MPLA) nanoparticles	365 ± 49*	849 ± 34*	1575 ± 61*	7007 ± 584*
HBcAg protein	(MPLA) nanoparticles	UD	159 ± 50	UD	UD
	HBcAg	UD	UD	UD	314 ± 20
	HBcAg + MPLA	UD	553 ± 27	UD	623 ± 67
	(HBcAg) nanoparticles	UD	NA	250 ± 12	NA
	(HBcAg+MPLA) nanoparticles	211 ± 37	1327 ± 22*	885 ± 16*	2828 ± 166*

UD: Below limit of the detection assay (156 pg/mL)

NA: Not applicable since the formulation was not included in the booster immunization regimen.

*Asterisk above indicating the difference between the treatment group means is statistically significant compared to other groups (S-N-K multiple comparison tests $\alpha=0.05$).

Table 2-1. Levels of IFN-γ secreted by primed T cells co-cultured with either HBc peptide or HBcAg protein. Lymph node and spleen cell samples from mice that were immunized with various vaccine formulations in either a single or a booster immunization.

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Chapter 3

Identification and Characterization of Pekin Duck MHC class II DRA and Invariant Chain Genes

3.1 Introduction

One of the limitations of using the duck hepatitis B virus (DHBV) model to study hepatitis B infection (HBV) is the lack of knowledge about the immune system of ducks. Antigen presenting cells (APCs) are critical initiators in the immune response to pathogens. Mechanisms to identify APCs are key to the development of cellular assays to characterize the immune response in ducks. In mammals, MHC class II molecules are expressed primarily on professional antigen presenting cells (APCs) such as B cells, macrophages and dendritic cells. Professional APCs express MHC Class II gene products constitutively, but the level of their expression is dependent on the activation status and developmental stage (1). Although MHC molecules are known to be highly polymorphic, the gene product encoded by the DRA gene is typically monomorphic or oligomorphic and the nucleotide sequence of DRA is well conserved even among different species (2-6). We have analyzed the MHC class II DRA gene as a first step in characterizing a marker to facilitate the isolation or identification of APCs from Pekin ducks (*Anas platyrhynchos*).

MHC class II associated-invariant chain (Ii) is a non-polymorphic type II transmembrane glycoprotein. In mammals, invariant chain plays a critical role in regulating MHC class II expression and function (7). Invariant chain forms a nonameric complex $(\alpha\beta\text{-Ii})_3$ with newly synthesized MHC class II $\alpha$and β chains in the endoplasmic reticulum (ER) to promote the folding of class II $\alpha$and β chains, stabilize the peptide-free class II $\alpha\beta$ heterodimers structure, and direct the localization of class II alpha/beta heterodimers to endosomes (8), where peptide loading occurs (7, 9). Via the class II-associated invariant chain peptide (CLIP) region of invariant chain, Ii associates with class II molecules to prevent the MHC class II molecules from binding inappropriate peptide prematurely (10). Four different invariant chain isoforms have been detected in humans (11, 12). Two forms of Ii (p33 and p35) are generated by the use of alternative translation initiation sites (13). Another two distinct isoforms (p41 and p43) are derived from alternative splicing of Ii exon 6b. Although p31 is the predominant form, antigen presentation is facilitated mainly by the minor form of Ii, p41 (14, 15). Recently, two invariant chain isoforms resulting from alternative splicing were identified in chicken, presumably the homologues of mammalian p33 and p41 (16).

Here we present the nucleotide sequence of MHC class II DRA and invariant chain of Pekin ducks. The regions important for the biological function of human MHC class II DRA and invariant chain are conserved in the Pekin duck sequences. The duck MHC class II DRA and invariant chain genes are strongly expressed in spleen and constitutively expressed in mononuclear cells isolated from either blood or spleen. In addition, an alternatively spliced invariant chain isoform was found in Pekin ducks. This longer Ii isoform was expressed at higher levels than the short one suggesting this is the predominant isoform in ducks. Phylogenetic analysis based on amino acid sequences showed that the MHC class II DRA and invariant chain of Pekin duck were most closely related to those of the chicken. Cloning these genes represents a first step toward development of markers to identify antigen-presenting cells in ducks.

3.2 Materials and Methods

3.2.1 Animal model

Pekin ducks (*Anas platyrhynchos*) were bred and housed at the Health Science Laboratory and Animal Care facility at University of Alberta (kindly provided by Dr. D.L. Tyrrell). The care and the use of laboratory animals complied with the Canadian Council on Animal Care (CCAC) guidelines. Animals were euthanized by an overdose 3-5 mL of Euthanyl (240 mg/mL) (MTC Pharmaceutical, Cambridge, ON, Canada) given intravenously.

3.2.2 Cloning of duck MHC class IIα and invariant chain

3.2.2.1 cDNA library construction

A cDNA library was constructed from mRNA isolated from the spleen of a Pekin Duck by Mesa et al (17). Clones encoding DRA and invariant chain were identified in an expressed sequence tag project (C. Radford and K. Magor, unpublished). The clones were subsequently excised *in vivo* from the λZAP Express vector using the ExAssist/XLOLR helper phage system (Stratagene). Briefly, phagemid particles were excised by coinfecting XL1-BLUE MRF' cells with ExAssist helper phage. The excised pBluescript phagemids were used to infect *E.Coli* XLOLR cells, which lack the amber suppressor necessary for ExAssist phage replication. Infected XLOLR cells with kanamycin resistance were selected. Resultant colonies contained the double-stranded phagemid vector with the cloned cDNA insert. A single colony was grown overnight in LB-kanamycin, and plasmid DNA was purified by minipreps according to standard protocol (18).

3.2.2.2 DNA sequencing

The cloned DNA was sequenced by automated fluorescent dye-terminator cycle sequencing, using the chain-termination dideoxynucleotide method of Sanger and coworkers (19). PCR was carried out to incorporate dideoxynucleotides which contain fluorescent dyes in a primer extension sequencing reaction using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Uppsala, Sweden). The automated sequencing was performed using the ABI Prism 377 DNA sequencer (Perkin-Elmer Applied Biosystems Inc. Foster City, CA). Both strands of the cDNA were sequenced using commercial T3 or M13 primers (5'-ATGGAGAACTTGGAGAAGCAGC-3' and 5'-CATTTTGCTGCCGGTCA-3'). A complete sequencing of the cDNA strands was obtained by walking the sequence in both directions with suitable primers (Table 3-1).

3.2.2.3 Sequence analysis

Complete cDNA sequences for invariant chain and DRA clones were obtained. Both sequences were determined at least twice for both strands. Analysis and assembly of data derived from DNA sequencing was performed using the PepTool™ program (20). GeneTool™ program was used to predict the putative open reading frame (ORF), and the hypothetical translation predicted a amino acid protein sequence (20). The EST sequences were submitted to the National Center for Biotechnology Information (NCBI) non-redundant database for TBLASTN and TBLASTX searches for matches to known sequences in GenBank. Matches with e-values of less than 1 x 10^{-4} were considered to be significant. The completed sequences were submitted to Genbank. An accession number assigned for duck DRA is AY905539. The accession number for the short invariant chain isoform is AY905540. The accession number for the invariant chain RT-PCR product containing the alternatively spliced exon sequence is AY905541.

3.2.3 Cell isolation and culture

A single-cell suspension from spleen was prepared by smearing cell aggregates between two frosted slides. Blood samples were collected in heparin-containing BD vaccutainers. Fractionation of mononuclear cells from the collected peripheral blood was achieved by using Ficoll-Pague™ Plus (specific gravity 1.077+0.001g/cm^3; Amersham Biosciences, Uppsala, Sweden) density gradient. The resulting mononuclear cells were resuspended (10^6 cells/mL) with RPMI media supplemented with 20% fetal calf serum, 2 mM L-glutamine and 100 U/mL penicillin and 100 μg/mL streptomycin. Macrophages were isolated as previously described (21). Briefly, the isolated mononuclear cells were cultured for 72 h by adherence to tissue culture dishes at 37° C in 5% CO_2 incubator. LPS-stimulated macrophages were treated with LPS in the media (1μg/mL) for 24 h. The non-adherent mononuclear cells were obtained by collecting the non-adherent cells in the media from the mononuclear cell culture after 24 hr incubation.

3.2.4 Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA samples were collected from the mononuclear cell culture using Qiagen RNeasy Mini Kit (Qiagen, Inc., Mississauga, ON, Canada). Single-stranded cDNA was synthesized from total RNA using Oligo (dT)20 primer using 5 μg of total RNA as template, incubated with ThermScript™ Rnase H$^-$ reverse transcriptase at 50°C for 45 minutes, as directed by the manufacturer's protocol (Invitrogen). Gene-specific oligonucleotide primers for Pekin duck MHC class DRA and invariant chain were designed based on the highly conserved regions. The RT-PCR products generated by these primers were also used as the templates for preparing ^{32}P-labelled MHC class IIα and ^{32}P-labelled Ii probe. The forward and reverse primer sequences used in the PCR for MHC class DRA were 5'-AACCGCTCGCAGGGCACCATCGC-3'and 5'-TCCCAGTGCGTCATCAGCGGCTC-3', respectively. The forward and reverse primers for the invariant chain were 5'-CCTGCCTCTCGCCCCCTCCCTAG-3', and 5'-GCGCCCAGCTTGAGCATGTCCAC-3', respectively. A set of β-actin primers (Forward 5'- ACCGCGCAACTCCCCGAAGCCAG-3' and Reverse 5'-ATAGCTGTCTTTCTGGCCCATGC-3') served as a control of amount and quality of cDNA. The PCR assay was carried out using the following conditions: 25 successive cycles of denaturation at 94 °C for 30 sec, primer annealing at 63 °C for 30 sec, and DNA extension at 72 °C for 1 min. PCR products were electrophoresed on a 0.8% agarose gel to detect the specific bands.

3.2.5 Northern Blot Analysis

Collections of total RNAs from various organs were obtained and purified using Trizol reagent according the manufacturer's protocol (Invitrogen). The purity and integrity of RNA was assessed by absorbance at 260/280 nm. The expression pattern of MHC class IIα and Ii were analyzed using Northern blot hybridization. The total RNA samples (10 μg/lane) were subjected to electrophoresis on a 0.6% formaldehyde, 1.2% agarose gel and transferred to a Nytran® supercharge nylon membrane (Schleicher & Schuell BioScience Inc., Keene, NH), followed by UV cross-linking (UV Stratalinker 2400, Strategene). The blot was prehybridized at 42 °C for 2 hours and then hybridized overnight with either ^{32}P-labelled MHC class II DRA or Ii probe. The 304 bp duck DRA probe and 341 bp invariant chain probe were labelled by random priming (22) using Prime-it II random primer labelling kit (Strategene). The isolated RT-PCR product derived from the reaction described above was used as the DNA template for the probe. The blot was washed once in 1X SSPE and 0.1% SDS at 65 °C for 10 min, followed by three washes in 0.1% SSPE and 0.1% SDS for 20 min each. Signal was detected by exposing the blot to Kodak X-Omat AR film at –80 °C for 2-3 days.

3.2.6 Phylogenetic analysis

The phylogenetic analysis was performed using the MEGA 3.0 program (23). The phylogenetic trees were constructed by the neighbor-joining method. The bootstrap percentages of the tree branching were obtained from 1,000 replicates.

3.3 Results

3.3.1 Characterization of MHC DRA and Ii cDNA sequences

3.3.1.1 Analysis of duck DRA cDNA and predicted polypeptide

Five cDNA clones identified from duck spleen had significant similarities to MHC class II DRA genes. We completely sequenced the cDNA clone 15.G9 in both directions. This sequence has been deposited in GenBank under the accession number AY905539. Analysis of the nucleotide sequence of clone 15.G9 revealed that it is a full-length cDNA sequence containing a 5'-untranslated region of 14 nucleotides (nt), a single open reading frame with 768 nt and a 3'-untranslated region of 509 nt with a classical polyadenylation signal (AATAAA) located 20 nt upstream from the poly (A) tail. It encoded a putative DRA molecule of 255 amino acids (aa) consisting of a 22-aa leader sequence, an 89-aa $\alpha 1$ domain, a 93-aa $\alpha 2$ domain, and a 51-aa connecting peptide/transmembrane/cytoplasmic tail domain (Figure 3-1). A search of the database with the amino acid sequence deduced from the cDNA 15.G9 revealed significant similarities to known MHC class II DR α chain genes (Figure 3-2). We named the clone *Anpl*-DRA*0101. The predicted amino acid sequence of the *Anpl*-DRA*0101 showed 51% identity (E value: $2e^{-64}$) with chicken B-LA (GenBank Accession NP_001001762) (2). The deduced protein of the *Anpl*-DRA*0101 contained the distinguishing sequence features of the MHC class II alpha chain. Conserved residues for the MHC class II alpha domain and immunoglobulin domain were present in *Anpl*-DRA*0101 based on the conserved domain searches (24). Of particular interest are the two asparagine residues at positions 88 and 95 (equivalent to HLA DRA residues 87 and 94) and an arginine residue at position 102 (residue 101 in HLA DRA) of the $\alpha 1$ domain, known to bind the main chain atoms of antigenic peptide in human DR1, were conserved in Pekin duck DRA (25). Other residues of the $\alpha 1$ domain involved in peptide binding in human DRA were almost identical to the predicted amino acid residues of duck DRA except for residue 29 and 94. Isoleucine at aa 29 and alanine at aa 94 in human DRA was replaced with leucine and glutamine in Pekin duck DRA, respectively. Such amino acid substitutions were identical to chicken B-LA in the corresponding positions.

An alignment of the inferred amino acid sequence of *Anpl*-DRA*0101 with those of cat, dog, human, rhesus monkey, goat, sheep, cow, mouse and chicken, revealed that conserved residues were evident throughout $\alpha 1$, $\alpha 2$, connecting peptide, transmembrane (TM) and cytoplasmic (CT) domains. For example, the two cysteine residues at duck DRA residue 133 and 189 (equivalent to HLA-DRA residue 132 and 188) (3) are highly conserved among different species suggesting that the cysteine residues in the alpha 2 domain are critical in forming the intramolecular disulfide bridge. In human and mouse, there are two N-linked glycosylation sites in the DR alpha chain, one located in $\alpha 1$ (asparagine residue 103 in HLA-DRA), and another one in $\alpha 2$ domain (asparagine residue 143 in HLR-DRA). However, only one potential glycosylation site at the corresponding position in the α1 domain was found in the Pekin duck sequence, while the second glycosylation site in α2 domain is lost. The loss of the glycosylation site in the α2 domain was also observed in the amino acid sequences of chicken and rhesus monkey (Figure 3-2). A phylogenetic tree was constructed to compare the relatedness of duck DRA and the DRA sequences of other species. The phylogenetic reconstruction of DRA genes showed the Pekin duck DRA was closely related to chicken B-LA, suggesting an orthologous relationship between these two genes (Figure 3-3).

3.3.1.2 Analysis of duck Ii cDNA and predicted polypeptide

One cDNA clone (1G1) isolated from duck spleen had significant similarity to invariant chain genes. We sequenced this clone completely and the sequence has been deposited in GenBank under the accession number AY905540. The nucleotide sequence of the clone 1G1 was 1443 bp and contained an open reading frame of 856 nucleotides, encoding a putative protein of 221 amino acids that has an estimated mass of 24 kDa (Figure 3-4). The sequence encoded by this clone was named duck Ii-1. A search of Genbank using TBLASTX with the duck Ii cDNA clone showed the greatest similarity to the chicken MHC class II-associated invariant chain (GenBank Accession AAT36345.1) with 68% amino acid identity (E value: $8e^{-78}$). It also showed amino acid identities of 51% (E value: $1e^{-39}$) to the cow invariant chain (GenBank Accession AAX46336) and 45% (E value: $1e^{-37}$) identity to human invariant chain (GenBank Accession NP_004346). The 3'-untranslated region of 763 nucleotides contained a polyadenylation signal (AATAAA) located 39 nt upstream from the poly(A) tail.

Using invariant chain gene-specific primers flanking exon 4 and exon 8, two bands were amplified by reverse-transcription PCR from cDNA prepared from macrophages generated from the peripheral blood mononuclear cells. The larger DNA fragment (530 bp) was189 bp longer than the expected product (341 bp). To determine the sequence of the amplified 530 bp product, it was extracted using the QIA quick gel extraction kit (Qiagen) and cloned into the pCR®2.1-TOPO® vector (TOPO TA cloning kit; Invitrogen). The longer Ii sequence was identical to the shorter Ii gene where they overlapped. They differed by the presence of an extra segment, which is located between nucleotides 599-787. This fragment encoded a cysteine-rich thyroglobulin domain (Tg) domain of 63 amino acids. This conserved Tg domain, corresponding to the alternative spliced region exon 6b, has been reported in chicken and other mammalian species (12, 16, 26). The addition of the alternatively spliced 63 amino acids to the duck invariant chain cDNA clone Ii-1 gives rise to a longer invariant chain isoform, named Ii-2.

An alignment of the predicted amino acid sequence of the duck Ii-1 clone with those of other species revealed the conservation of distinct features required for invariant chain function (Figure 3-5). These include the leucine-8 isoleucine-9 motif responsible for endosomal/lysosomal sorting in the cytoplasmic tail at the N-terminal (31 aa), the hydrophobic transmembrane domain (24 aa), the class II associated invariant chain peptide (CLIP) and Ii trimerization segment (aa 120-195) in the luminal region (166aa) responsible for interaction with MHC class II glycoproteins. Amino acid alignment of the transmembrane (TM) domain and the Thyroglobulin type-1 (Tg) domain between duck and chicken invariant chain (16) demonstrated that they were highly conserved in these regions (Figure 3-6).

3.3.2 Phylogenetic analysis

Phylogenetic trees were constructed to compare the relatedness of duck invariant chain with amino acid sequences of other species. The phylogenetic tree showed the expected clustering, confirming the highest similarity between Pekin duck and chicken invariant chain (Figure 3-7). The high bootstrap values also lend support for the relationship between these sequences.

3.3.3 Tissue distribution

To determine sites of expression of duck DRA and duck Ii genes, northern blot analyses were performed (Figure 3-8). The expression of both the duck DRA and Ii was much higher in the spleen, lung, intestine and liver on the northern blot, compared to the heart or kidney. In particular in the spleen, both DRA and Ii were strongly expressed. A single band of a ~1.2 kb size was detected for the duck DRA transcript. Two bands with transcript sizes of ~1.7 kb and ~1.5 kb were detected for invariant chain. The size of the smaller band coincided with that of the size of the cDNA clone encoding the Ii-1 transcript (1443 bp). The larger band (~1.7 kb) likely corresponding to the isoform of invariant chain that contained the thyroglobulin domain, detected by reverse transcription PCR. Both invariant chain transcripts were expressed at about the same level in lung, kidney and intestine, while the larger transcript (~1.7 kb) was expressed at higher levels in the spleen and liver.

3.3.4 MHC class II DRα and the associated invariant chain mRNAs were expressed by macrophages

To determine whether duck adherent mononuclear cells express MHC class II DRA and Ii, RT-PCR using gene specific primers was used. Putative macrophages were generated from either spleen or blood-derived monocytes by adherence on tissue culture flasks for 72h. The non-adherent mononuclear cells consisting mainly of B cells, T cells and thrombocytes were also tested for expression of DRA and Ii. Cultured macrophages expressed duck DR alpha and Ii constitutively (Figure 3-9). DRA and Ii expression was detected in both adherent and the non-adherent mononuclear cells, whether they were isolated from blood or spleen.

To examine the expression of DRA and Ii upon activation of adherent mononuclear cells, we cultured the macrophages in the presence of LPS. RNA was isolated from adherent mononuclear cells at various time points after exposure to LPS and DRA expression was analyzed by northern blot (Figure 3-10). Macrophages exposed to LPS for 12 hours produced slightly less duck DR alpha transcript. The expression of DRA expression is further reduced after a 24-hour LPS incubation period.

3.4 Discussion

Ducks are an important animal model for hepatitis B research, however the tools necessary to study processing and presentation of antigens for vaccine development are lacking. We have characterized two genes expressed by antigen presenting cells to serve as markers for identifying antigen presenting cells of ducks, MHC class II DRA and two isoforms of invariant chain Ii. We have characterized the expression of DRA and Ii transcripts in tissues and cells involved in antigen presentation.

While class II MHC genes have been analyzed extensively in mouse and human since their identification (27), relatively little information is available for duck. The MHC class II DRA cDNA sequence (Anpl-DRA*0101) from the Pekin duck (*Anas platyrhynchos*) has two distinct features that are hallmarks of MHC class II DR alpha: conserved residues in the alpha 1 domain and the immunoglobulin domain. The putative peptide binding region sequence of duck MHC class II α are almost identical to the DRA sequences of chicken and mammals (2, 25, 28). In human and mouse, MHC class II molecules are constitutively expressed on professional APCs i.e., B cells, dendritic cells, and macrophages, and in specialized thymic epithelium (29). Expression profiles of duck MHC class II DRA and Ii determined by RT-PCR analyses indicate that duck macrophages express MHC class II DR alpha and invariant chain constitutively. Other mononuclear cells consisting of B cells, T cells and thrombocytes, collectively classified as non-adherent mononuclear cells, are also shown to express MHC class II DRA and invariant chain. Consistent with the expression profile of MHC class II molecules, the duck MHC class II DRA transcript occurs in greatest abundance in the major lymphoid tissue (spleen) determined by Northern blot analysis. Overall, the deduced amino acids from *Anpl*-DRA*0101 bear sequence homology to chicken B-LA and the DRA of other mammalian species. The fact that *Anpl*-DRA*0101 gene product is found in great abundance in the lymphoid tissue and has a close genetic relationship with chicken B-LA further support that Anpl-DRA*0101 is a MHC class II DR alpha homologue in Pekin duck.

Exposure of duck macrophages to LPS for 24 h caused a significant reduction of transcript corresponding to MHC class DR alpha. There is evidence that MHC class II synthesis and surface expression is reduced after prolonged (18 h) treatment of mouse macrophages with LPS (30) or when mouse macrophages were simultaneously treated with LPS and IFN-γ (31, 32). The inhibitory effect of LPS on MHC class II transcript expression has been proposed to be a result of increased levels of TNF-α or nitric oxide secretion (33). *In vitro*, LPS may either inhibit or enhance MHC class II expression depending upon the sequence of the IFN-γ and LPS signals encountered by macrophages (33). As the level of surface expression of class II molecules correlates with the ability of cells to present antigen to T cells and the vigor of the immune response (34), we need to determine whether transcript levels correlate with surface expression of MHC class II.

Evidence has shown that chicken and human invariant chains share both sequence and functional homologies (35). The high nucleotide sequence identity between Pekin duck and chicken invariant chain and their close genetic sequence relationships from the phylogenetic studies suggest that they are direct orthologues. The predicted amino acid sequence of the duck Ii bears the distinct features of the known invariant chains, which impart different functional properties in MHC class II antigen processing (36). The predicted amino acid sequence from Duli-1 revealed an open reading frame of 221 aa for the duck Ii cDNA, compared to 223 aa in chicken Ii. The first 10 amino acid residues at the N-terminal of the Ii cytoplasmic domain are identical between Pekin duck and chicken invariant chains. The leucine-isoleucine pair at positions 7 and 8 representing one of the leucine-based sorting signals in chicken (35) and human (37, 38) is conserved in duck Ii. The sorting signal within the cytoplasmic tail is important as it targets and directs the MHC class II molecules to the endocytic pathway for loading peptides derived from the exogenous source (39). The second leucine-based sorting signal (aa 18) described in chicken invariant chain is absent in Pekin duck. There is evidence that a single leucine signal is sufficient for endosomal sorting (40). Mutation of either one of the leucine signals in chicken invariant chain did not abort the endosomal targeting process (35). Duck invariant chain may rely on only one leucine-based signal for sorting or use other sequences to direct the intracellular trafficking of MHC class II.

Overall, duck invariant chain gene encodes a putative polypeptide with characteristic conserved residues that are critical for the function of an invariant chain. The class II-associated invariant chain peptide (CLIP) region, amino acids 80-106 in Pekin duck, aligned with amino acids 81-104 of human Ii (41) and amino acids 81-107 of chicken Ii (35). The CLIP region contains the peptide sequence required for binding to the class II-binding groove and for efficient Ii-class II complex formation (42). The two tryptophan residues at positions 168 and 172 located in exon 6 of mammal Ii essential for the formation of the Ii trimer (43, 44) are also present in duck Ii (aa 170 and 174 in Pekin duck). The Ii chain in mammals has two N-linked glycosylation sites within the trimerization region (aa 119 and aa 125 in human). However, like chicken invariant chain, Pekin duck Ii only has the second glycosylation site at aa 123.

Four different forms of invariant chain in humans result from alternative translation start sites or alternative splicing, namely, p33, p35, p41 and p43 (36, 45). Isoforms p35 and p43 are longer than p33 and p41 by 17 amino acids, respectively, due to the differential use of the first and the second in-phase start codon. In Pekin duck, only two Ii transcripts were found. The first in-phase start codon is missing in duck Ii. The 5' end of the putative amino acid residues of the Pekin duck Ii gene align with the second methionine of the mammalian Ii. The predicted amino acid sequences of the two Ii isoform from Pekin duck were similar to each other where they overlapped. They differed only by the presence or absence of 63 amino acids, located between exon 6 and exon 7. This region corresponds to the highly conserved cysteine-rich thyroglobulin (Tg) type-I domain, encoded by an alternatively spliced exon 6b found in mammalian cells (46). The thyroglobulin domain of the p41 form of Ii in human has been shown to exhibit a potent inhibitory activity against cathepsin L (47). It has been suggested that this conserved region possibly plays a role in regulating the proteolytic enzymes generating antigenic peptides. Two Ii isoforms were also found in chicken. The long Ii isoform in chicken containing this thyroglobulin domain (Ii-2) has also been reported recently (16). The longer duck Ii-2, like chicken Ii-2, is homologous to the human p41 Ii isoform.

Northern blot analysis showed low Ii expression in the heart and kidney, while relatively high expression of both Ii isoforms was detected in the spleen, lung, liver and intestine. The DuIi-1 and DuIi-2 encoded a short invariant chain isoform and a long invariant chain isoform, which are potentially the homologues of p33 and p41 of human invariant chains, respectively. The two isoforms appeared be differentially expressed in various duck tissues, with the long Ii isoform predominantly expressed in the spleen and liver. The human p41 Ii isoform has been shown to regulate proteolytic activity in endosomal compartments (48-50), facilitate the peptide-loading process (51) and enhance antigen presentation (14). In mouse, the relative expression the invariant chain isoforms depends on cell type. The proportion of p41 in relation to p31 varied from 9% in B cells to 43% in epidermal Langerhans cells (52). The differential expression pattern of the invariant chain isoforms in duck tissues may reflect different cell populations and/or immune activities in these organs.

We have determined the DNA sequences of the expressed MHC class II DRA and invariant chain genes of Pekin duck. In mammals, MHC class II molecules and invariant chains play a key role in processing and presenting antigen peptides to T cells for the initiation of an immune response. We found that the deduced amino acid sequences of both MHC class DRA and invariant chain in Pekin duck were highly conserved when compared with those of the chickens and mammals. The conservative nature of these molecules suggests positive pressure on these genes. The overall lack of sequence variation in the putative peptide-binding region of the DRA genes among different species implies a functional role for this molecule in recognizing and generating pathogen-specific immune response. A recent study reported that monoclonal antibodies to IgL and CD3, CD4 and CD8 antigens of Pekin ducks have been developed to identify T cells and B cells in ducks (21), however, reagents to differentiate duck monocytes/ macrophages from thrombocytes in the peripheral blood are still unavailable. Since MHC class II molecules are primarily expressed by antigen presenting cells, the clones and sequence information provided in this study may be useful for generating antibodies to duck DR alpha, which will allow us to separate duck monocytes/macrophages from duck thrombocytes and examine the function of these cells.

```
     5'UTR
   1 gtgtcacaga ggtg

     Signal sequence
      M   A   T   G   R   G   I   P   L   V   L   L   A   V   L   A   L   Q   G   S
  15 atg gcc aca gga cgg ggc atc ccg ctg gtg ctg ctg gcc gtg ctg gcc ctg cag ggc tcg
      G   A
  75 ggg gcc

     Alpha 1 domain
      V   K   V   G   H   V   L   M   Q   T   E   F   Y   Q   R   D   E   T   R   N
  81 gtg aaa gtg ggc cac gtg ctg atg cag act gaa ttc tac cag cgg gat gag acg cgg aac
      K   E   G   G   Q   F   M   F   D   F   D   G   D   E   I   F   H   V   D   L
 141 aaa gag ggc ggc cag ttc atg ttc gat ttc gat ggg gat gag atc ttc cac gtg gac ctg
      E   K   S   E   T   I   W   R   L   P   I   F   T   T   F   T   S   F   E   A
 201 gag aag tcg gag acc atc tgg cgc ctg ccc atc ttc acc acc ttt acc agc ttc gag gcc
      Q   G   A   L   Q   N   I   A   V   D   K   Q   N   M   E   I   M   M   K   R
 261 cag ggg gcc ctg cag aac atc gcc gtg gac aag cag aac atg gag atc atg atg aag agg
      S   N   R   S   Q   G   T   I   A
 321 tcc aac cgc tcg cag ggc acc atc gcg

     Alpha 2 domain
      P   P   E   V   T   V   F   S   E   D   P   V   E   L   G   D   P   N   I   L
 348 cca ccc gag gtg acg gtg ttc tcc gag gac ccc gtg gag ctg ggg gat ccc aac atc ctc
      I   C   Y   V   D   K   F   W   P   S   V   I   T   I   T   W   M   K   N   G
 408 atc tgc tac gtg gac aag ttc tgg ccg tcc gtc atc acc atc acc tgg atg aag aac ggg
      Q   E   V   T   E   G   I   S   E   T   V   F   Y   R   Q   A   D   N   G   F
 468 cag gag gtg acg gag ggc atc tcc gag acc gtc ttc tac cgc cag gca gac aac ggc ttc
      Y   K   F   S   Y   L   P   F   I   P   T   R   G   D   Y   Y   D   C   R   V
 528 tac aag ttc tcc tac ctg ccc ttc atc ccc acg cgg ggc gac tac tac gac tgc cgt gtg
      E   H   W   G   L   R   E   P   L   M   T   H   W
 588 gag cac tgg ggg ctg cgc gag ccg ctg atg acg cac tgg

     Connecting peptide
      E   P   Q   V   P   L   P   V   S   E   S   T   E
 627 gag ccc cag gtg ccc ctc ccc gtc tcc gag agc acc gag

     Transmembrane region
      T   L   V   C   A   L   G   L   A   V   G   I   V   G   I   V   V   G   T   I
 666 acc ctg gtg tgc gcc ctg ggc ctg gcc gtg ggc atc gtt ggc atc gtc gtg ggc acc atc
      L   I   I
 726 ctc atc atc

     Cytoplasmic region
      K   A   M   K   M   N   N   A   R   N   Q   R   G   L   L   *
 735 aag gcc atg aag atg aac aac gcc cgc aac cag cgg ggc ctc ttg tga

     3'UTR
 783 ggaggcagag gccgggcctc cccccagccg gaccctgtgc cccttcagcc acccccttgc ttcagccccc
 853 ccctgccaag agctgctcgc tcctgtgccc cacatcggtg ccgcgcctcc ccagagcttc cttctcccac
 923 acatgctgca tgaagacgcc cctcaggctg ctccggggtg gctgaattcc caccccacaa cgggatgggt
 993 ttgagcagag gtagcccccg gggccacccc gagttgcccg ctctcagggg acatcgccgc accgcctgcg
1063 ctggtcccag catccagcat gcgccacgtc ttctcctcgc ccagatgcgg gggtctcttt tgagggagac
1133 ctttggggtg agcgggggag ggtcctgccc cccggcttcc ccctggcacc cagttccatg cccgccactc
1203 ctcctctgct gttctgacac tgcaataaag tggattgggg ttgaaaaaaa aaaaaaaaaa aaaaaaaaaa
1273 aaaaaaaaaa aaaaaaaaa
```

Figure 3-1. The nucleotide and the predicted amino acid sequences of Pekin duck MHC class II DRA. Borders of each domain were assigned based upon sequence homology between duck DRA and other DRA homologous sequences.

```
                                                 p p p                p p p    p
Pekin duck DRA MATGRGIPL- --VLLAVLAL QGSGAVKVGH VLMQTEFYQR DETRNKEGGQ FMFDFDGDEI  60
Chicken B-LA   ..VLS.AAVP LLL.GVLGGV G---..LKP. ..L.A..... S.GPD.AWA. .G.H..A..L  60
Cat DRA        .TINGVLV.G FLIVAFLMCP .E...I.EE. .II.A...LK PDSS----.E ..........  60
Dog DRA        .TISGVPV.G FFIMAFLMGP .E.W...EE. .II.A...LT PDPS----.E ..........  60
Human DRA      ..ISGVPV.G FFIIAVLMSA .E.W.I.EE. .II.A...LN PDQS----.E ..........  60
Rhesus monkey  ..ESGVPV.G FFIIAVLMSA .E.W.I.EE. .II.A...LN PDQS----.E ..........  60
Goat DRA       ..IT.VPI.G LFIT-VLIS. .ELW.I.ED. .II.A...LN P.QS----AE ..........  60
Sheep DRA      ..IT.VPI.G LFIT-VL.S. .E.W.I.ED. .II.A...LN P.ES----AE ..........  60
Cow DRA        ..IT.VPI.G LFIT-VLIG. .E.W.I.EN. .II.A...LK P.ES----AE ..........  60
Mouse H2-Ea    ...IGALL.R FFFIAVLMSS .K.W.I.EE. TII.A...LL PDK.----.E ..........  60
               p           p       p  ppp  p    p  p  pp   p   p n
Pekin duck DRA FHVDLEKSET IWRLPIFTTF TSFEAQGALQ NIAVDKQNME IMMKRSNRSQ GTIAPPEVTV 120
Chicken B-LA   ...E.DAAQ. V....E.GR. A......... .M..G...L. V.IGN..... QDFVT..LAL 120
Cat DRA        ....M..K.. V...EE.GR. A........A ......A.LD .LI....NTP N.NE...... 120
Dog DRA        ....M..K.. V...EE.GR. A........A ......A.LD T.I....HTP N.NV...... 120
Human DRA      ....MA.K.. V...EE.GR. A........A ......A.L. ..T....YTP I.NV...... 120
Rhesus monkey  ....MA.K.. V...EE.GR. A........A ......A.L. ..T....NTP I.NV...... 120
Goat DRA       ....MQ.K.. V....E.GR. A........A .M..M.A.LD ..I....NTP N.NV.....L 120
Sheep DRA      ....MQ.K.. V....E.GR. A........A .M..M.A.LD ..I....NTP N.NV.....L 120
Cow DRA        ....MG.K.. V....E.GH. A........A .M..M.A.LD ..I....NTP N.NV.....L 120
Mouse H2-Ea    ....I..... ....EE.AK. A........A ......A.LD V.KE...NTP DANVA..... 120

Pekin duck DRA FSEDPVELGD PNILICYVDK FWPSVITITW MKNGQEVTEG ISETVFYRQA DNGFYKFSYL 180
Chicken B-LA   .PAEA.S.EE ..V....A.. ...P.A.ME. RR..AV.S.. VYDS.Y.GRP .LL.R..... 180
Cat DRA        L.NS.....E ......FI.. .S.P..NV.. LR..KP..T. V.....LPRE .HL.R..H.. 180
Dog DRA        L.NT.....E ......FI.. .S.P..NV.. LR..NP..T. V...I.LPRE .HL.R..H.. 180
Human DRA      LTNS....RE ..V...FI.. .T.P.VNV.. LR..KP..T. V.....LPRE .HL.R..H.. 180
Rhesus monkey  LTNS.....E ..V...FI.. .S.P.VKV.. L...KP..T. V.....LPRE .HL.R..H.. 180
Goat DRA       LPNK.....E ..T...FI.. .S.P..SV.. LR..IP..D. V.Q...LPRD .HL.R..H.. 180
Sheep DRA1     LPNK..Q..E ..T...FT.. .S.P..SV.. LR..IP..D. V.Q...LPRD .HL.R..H.. 180
Cow DRA        LPNK.....E ..T...FI.. .S.P..SV.. LR..KP..D. V.Q...LPRN .HL.R..H.. 180
Mouse H2-Ea    L.RS..N..E ......FI.. .S.P.VNV.. FR..RP.... V.....LPRD .HL.R..H.. 180

Pekin duck DRA PFIPTRGDYY DCRVEHWGLR EPLMTHWEPQ VPLPVSESTE TLVCALGLAV GIVGIVVGTI 240
Chicken B-LA   ..V.Q...V. S.A.R...AE G.VQRM...E ..E.P...SA ..W..V.... ..A..AA..A 240
Cat DRA        ..L.SAE.V. ..K......D ...LK...FD A.T.LP.T.. NV......V. .L...I.... 240
Dog DRA        ..L.SAE.V. ..K......D ...LK...FE P.T.LP.T.. NV......I. .L...IT... 240
Human DRA      ..L.STE.V. .........D ...LK...FD A.S.LP.T.. NV......T. .L...II... 240
Rhesus monkey  ..L.STE.I. ..K......D A..LK...FD A.S.LP.T.. NV......I. .L...I...V 240
Goat DRA       ..L..TE.V. ..K......N ...LK...YE A.A.LP.T.. NA......I. AL...IA... 240
Sheep DRA1     ..L..TE.V. ..K......N ...LK...YE A.A.LP.T.. NA......I. AL...IA... 240
Cow DRA        ..L..TE.V. ..K...L..N ...LK...YE A.A.LP.T.. NA......I. AL...IA... 240
Mouse H2-Ea    T.L.STD.F. ..E.D....E ...RK...FE EKTLLP.T.. NV......F. .L......I. 240

Pekin duck DRA LIIKAMKMNN ARNQR-GLL 259
Chicken B-LA   ..LR.VRR-. .A.RQP... 259
Cat DRA        F...G.RKV. .GER.-.A. 259
Dog DRA        F...G.RKVK .GER.-.P. 259
Human DRA      F...GLRKS. .AER.-.P. 259
Rhesus monkey  F...GVRKS. .AER.-.P. 259
Goat DRA       F...GVRKA. TVER.-.P. 259
Sheep DRA1     F...GVRKA. TVEG.-.P. 259
Cow DRA        F...GVRKA. TVER.-.P. 259
Mouse H2-Ea    ..M.GI.KR. VVER.Q.A. 259
```

Figure 3-2. An alignment of the predicted amino acid sequence of Pekin duck MHC class II DRA with the DRA sequences of other species using CLUSTAL W 1.8 program. Identities are indicated by a dot; gaps by a dash. The small letter n and p indicate the positions of the predicted N-linked glycan attachment site and peptide-binding site, respectively.

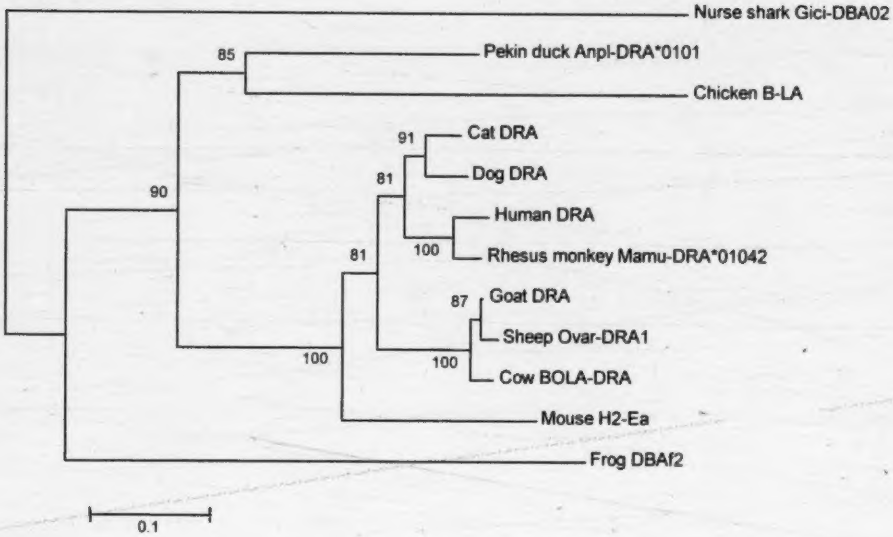

Nurse shark Gici-DBA02
85
Pekin duck Anpl-DRA*0101
Chicken B-LA
91
Cat DRA
81
Dog DRA
90
Human DRA
81
100
Rhesus monkey Mamu-DRA*01042
87
Goat DRA
Sheep Ovar-DRA1
100
100
Cow BOLA-DRA
Mouse H2-Ea
Frog DBAf2
0.1

Figure 3-3. Phylogenetic tree of deduced amino acid sequence of a full-length duck DRA sequences. Number at each node indicates the percentage of bootstrapping of a 1000 replications. Sequences included in the phylogenetic tree were obtained from the NCBI database at www.ncbi.nlm.nih.gov/.

```
     5' UTR            Cytoplasmic domain
                   M   A   E   E   Q   R   D   L   I   S   D   R   G   S   G   V
   1 cgacggtgcagcc atg gcc gag gag cag cgg gac ctc atc tcc gac cgc ggc agt ggg gtg
                                                               Transmembrane domain
      V   P   M   G   D   S   Q   R   S   A   F   G   R   R   A   A   L   S   T   L
  62 gtc ccc atg ggg gac agc caa agg tct gcc ttt ggg cgc aga gcc gcc ctg tcc acg ctg
                                                                                >>>
      S   I   L   V   A   L   L   I   A   G   Q   A   V   T   I   Y   F   V   Y   Q
 122 tcc atc ctg gtg gcc ctg ctg atc gcc ggc cag gct gtc acc atc tac ttc gtg tac cag
     Luminal region
      Q   S   G   Q   I   S   K   L   T   R   T   S   Q   N   L   Q   L   E   A   L
 182 cag agc ggg cag atc agc aag ctg acc agg acc tcc caa aac ctg cag ctg gag gcg ttg
                 ----------------------CLIP--------------------------------------
      Q   R   K   L   P   K   S   S   K   S   A   G   N   M   K   M   S   M   V   N
 242 cag cgc aag ctg ccc aag agc agc aag tcc gcc ggc aac atg aag atg tcg atg gtg aac
     ---------------------------------------
      T   P   L   A   M   R   V   L   P   L   A   P   S   L   D   D   T   P   V   K
 302 acc ccc ctg gcc atg agg gtc ctg cct ctc gcc ccc tcc cta gac gac aca ccc gtg aag
                 Trimerization domain
      D   M   G   P   P   S   N   K   T   E   D   Q   V   R   H   L   L   L   A   D
 362 gac atg ggg ccc ccc agc aac aag acc gag gac caa gtc agg cac ctg ctg ctg gca gac

      P   K   K   M   F   P   E   L   K   D   S   L   L   G   N   L   K   S   L   K
 422 ccg aag aag atg ttc ccg gag ctg aag gac agc ctg ctg ggc aac ctc aag agc ctg aag

      K   T   M   T   D   A   D   W   K   S   F   E   S   W   M   H   K   W   L   L
 482 aag acc atg acc gac gcg gac tgg aag tcc ttc gag tcc tgg atg cac aag tgg ctg ctg

      F   E   M   A   K   S   P   K   P   D   E   R   K   A   I   P   A   E   K   V
 542 ttc gag atg gcc aaa agc ccg aag ccg gac gag cgt aaa gcc atc cca gcg gag aaa gtg
     Thyroglobulin repeats
      Q   T   K   C   Q   A   E   A   N   F   G   G   V   H   P   G   R   F   R   P
 602 caa act aag tgc cag gca gag gcc aat ttc ggt ggt gtc cat ccg ggt cgc ttc cgc ccc

      E   C   D   E   N   G   D   Y   L   P   K   Q   C   H   A   G   T   G   Y   C
 662 gag tgc gat gag aac ggg gac tac ctg ccc aag cag tgc cac gcc ggc acg ggc tac tgc

      W   C   C   Y   K   N   G   T   K   I   E   G   T   A   T   R   G   E   L   D
 722 tgg tgc tgc tac aaa aac ggc acc aag att gag ggc acg gcc acc cgg gga gag ctg gac

      C   S   G   A   A   L   T   E   P   D   E   M   I   F   S   G   V   D   M   L
 782 tgc tcg ggg gct gca ctg acg gag ccc gac gag atg atc ttc tcc ggg gtg gac atg ctc

      K   L   G   A   E   K   A   K   *   3' UTR
 842 aag ctg ggc gct gag aaa gcc aag tag aagaggacacctgcagctgcccacacggctttcagcactccagc
 913 tcgtttttttttttttgctctatcccctttctgctcttccctcgctttcccaaacaccccccggcactccttcccagtga
 993 tcgcttcccctcccctgctccagggcctggcctggggtcgatgagagttggctggggctgtccccaggataaatgctaca
1073 gaggaaacgtttcctaaaactggaggtggagcaggctggaagcattttcagccgcagcagatccccagtcccttcccctg
1153 ttgctggtggggtacgagctgcagtgagggcgttgtgtgaaggacaccagctggttcccagccctgtgcctggcccccag
1233 ggcacttctcccccagcccaggctctgcgattgctcctgctgaagatcgagccactaatgcctgctttcttagcctgaac
1313 aaacattaaacgcagcaaaaccacctccaccagcttcttaactaaaccctctagacaggggctctactaaagcttcctat
1393 ttctcgtgtttattttttaatactcagttttgtagggataggtgctcaccttcagcttctcccttcagggtggggatca
1473 gcagcaggaggtagcagtgggaatgggagccaggcctggaggcagaggggcttttaatcttcctgaccgatctgatactt
1553 ccttgataaatgatgtcacaagctaattatttcaaataaactttgaataagaaagcctcagaaaaaaaaaaaaaaaaaa
```

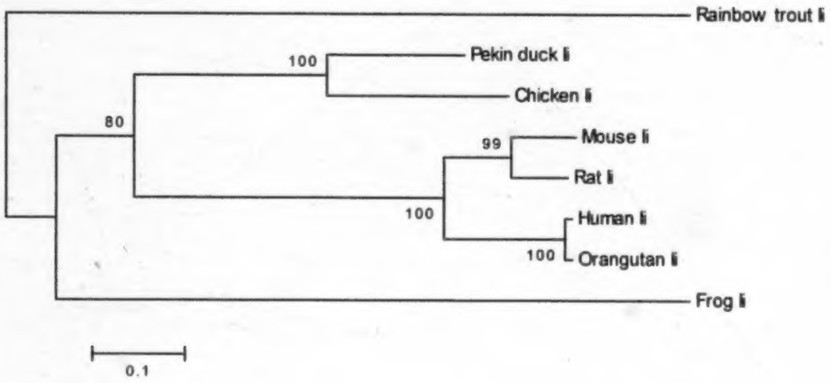
Figure 3-4. The nucleotide and the predicted amino acid sequences of the Pekin duck invariant chain isoform, Duli-2. Borders of each domain were assigned based upon sequence homology between duck other homologous Ii sequences. The additional nucleotide sequence, seen only in the Ii-2 isoform, is in bold.

```
Pekin duck Ii ---------- -----MAEEQ RDLISDRGS- GVVPMG-DSQ RSAFGRRAAL STLSILVALL  60
Chicken Ii    ---------- -----..... .....SD..S ..L.I.-N.E ..SL...T.. .A........  60
Mouse Ii      ---------- ------MDD. .....NHEQL PILGNRPREP E-RCS.G.LY TGV.V.....  60
Human Ii      MHRRRSRSCR EDQKPVMDD. .....NNEQL PMLGRRPGAP E.KCS.G.LY TGF....T..  60
Orangutan Ii  MHRRRSRSCR EDQKPVMDD. .....NNEQL PMLGRRPGAP E.KC..G.LY TGF....T..  60
Rat Ii        ---------- ------MDD. .....NHEQL PILGQRARAP E.NCN.GVLY TSV.V.....  60

Pekin duck Ii IAGQAVTIYF VYQQSGQISK LTRTSQNLQL EALQRKLPKS SKSAGNMKMS MVNTPLAMRV 120
Chicken Ii    .........Y .......... ..K...T.K. .S....M.IG TQP.NK.S.. TM.M.M..K. 120
Mouse Ii      L....T.A.. L...Q.RLD. ..I....... .S.RM..... A.PVSQ.R.A T---..L..P 120
Human Ii      L....T.A.. L...Q.RLD. ..V....... .N.RM....P P.PVSK.R.A T---..L.QA 120
Orangutan Ii  L....T.A.. L...Q.RLD. ..V....... .N.RM....P P.PVSK.R.A T---..L.QA 120
Rat Ii        L....T.A.. L...Q.RLD. ..V....... .N.RM..... A.PVSP.R.A T---..L..P 120

Pekin duck Ii LPLAPSLDDT PVKDMGPPSN KTEDQVRHLL L-ADPKKMFP ELKDSLLGNL KSLKKTMTDA 180
Chicken Ii    ......VG.M .MEA.E.R.. .....I.... .KS..R.T.. D...DM.... .R.....SAM 180
Mouse Ii      MSMD-NMLLG ...NVTKYG. M.Q.H.M... TRSG.LE-Y. Q..GTFPE.. .H..NS.DGV 180
Human Ii      ..MG-A.PQG .MQNATKYG. M...H.M... QN...L.VY. P..G.FPE.. RH..N..ETI 180
Orangutan Ii  ..MG-A.PRG .MQNATKYG. M...H.M... QN...L.VY. P..G.FPE.. RH..N..ETI 180
Rat Ii        .SMD-NMLQA ...NVTKYG. M.Q.H.M... TKSG.VN-Y. Q..G.FPE.. .H..NS.NGL 180
                                                  ↓
Pekin duck Ii DWKSFESWMH KWLLFEMAKS PKPDERKAIP AEKGAALT-- ---EPDEMIF SGVDMLKLGA 240
ChickenIi     ..QD..T... .........G ..ME.QNT.. ...AP.P.QP PSA..E.V.. .....V.AK- 240
Mouse Ii      N..I.....K Q......S.N SLEEKK---. T.APPKEPLD MEDLSSGLGV TRQELGQVTL 240
Human Ii      ...V...... H......SRH SLEQK----. TDAPPKESLE LEDPSSGLGV TKQ.LGPVPM 240
OrangutanIi   ...V...... H......SRH SLEQK----. T.APPKESLE LEDPSSGLGV TKQ.LGPVPM 240
Rat Ii        ...V.....K Q......S.N SLEEKQ---. TQTPPKEPLD MEDPSSGLGV TKQ..GQMFL 240

Pekin duck Ii EKAK 244
Chicken Ii    ---- 244
Mouse Ii      ---- 244
Human Ii      ---- 244
Orangutan Ii  ---- 244
Rat Ii        ---- 244
```

Figure 3-5. An alignment of the predicted amino acid sequence of the Duli-1 cDNA sequence with related sequences from the GenBank database using CLUSTAL W 1.8 program. Identities are indicated by a dot; gaps by a dash. An arrow indicates the position of the alternatively spliced exon 6b.

```
  Consensus VQTKCQAEAxFGGVHPGRFRPECDENGDYLPKQCxAxTGYCWCCYKNGTxIEGT 54
Pekin duck VQTKCQAEANFGGVHPGRFRPECDENGDYLPKQCHAGTGYCWCCYKNGTKIEGT 54
    Chicken VQTKCQAEASFGGVHPGRFRPECDENGDYLPKQCYASTGYCWCCYKNGTRIEGT 54

  Consensus ATRGxLDC                                               62
Pekin duck ATRGELDC                                               62
    Chicken ATRGQLDC                                               62
```

Figure 3-6. Alignment of the thyroglobulin type-1 (Tg) domain of the duck Ii-2 and chicken Ii-2 invariant chain. Identities are shown in shaded boxes.



Rainbow trout Ii
Pekin duck Ii
Chicken Ii
Mouse Ii
Rat Ii
Human Ii
Orangutan Ii
Frog Ii
100
80
99
100
100
0.1

Figure 3-7. Phylogenetic tree of deduced amino acid sequence of a full-length duck Ii sequence. Number at each node indicates the percentage of bootstrapping of a 1000 replications. Sequences included in the phylogenetic tree were obtained from the NCBI database at www.ncbi.nlm.nih.gov/.

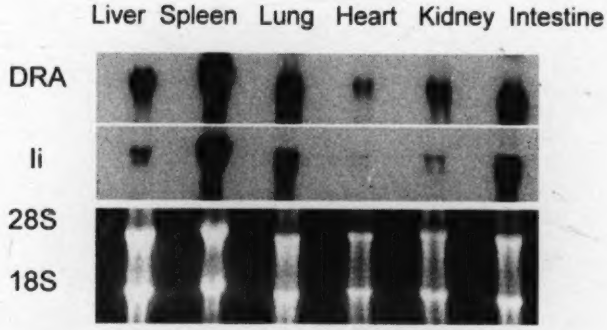

Liver
Spleen
Lung
Heart
Kidney
Intestine
DRA
Ii
28S
18S

Figure 3-8. Tissue expression of duck MHC class II DRA and invariant chain transcripts. Northern blot data indicated transcript sizes of around ~1.2 kb for DRA, and ~1.7 kb the Ii-2 isoform and ~1.5 kb for the Ii-1 isoform. Total RNA (10 μg) isolated from liver, spleen, lung, kidney and intestine was loaded from left to right. The original agarose gel stained with ethidium bromide demonstrated the relative amounts of RNA loaded in each lane.

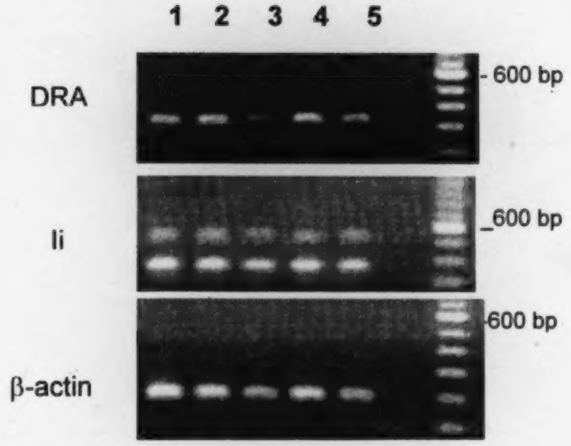


Figure 3-9. RT-PCR analyses of duck DRA and Ii gene expression in mononuclear cells isolated from blood and spleen. Lane 1. Non-adherent mononuclear leukocytes from blood; Lane 2. Adherent cells (macrophages) derived from blood monocytes; Lane 3. Adherent cells (macrophages) stimulated with LPS (1μg/mL) for 24 hours; Lane 4. Non-adherent mononuclear leukocytes from spleen; Lane 5. Adherent cells (macrophages) derived from spleen cells.

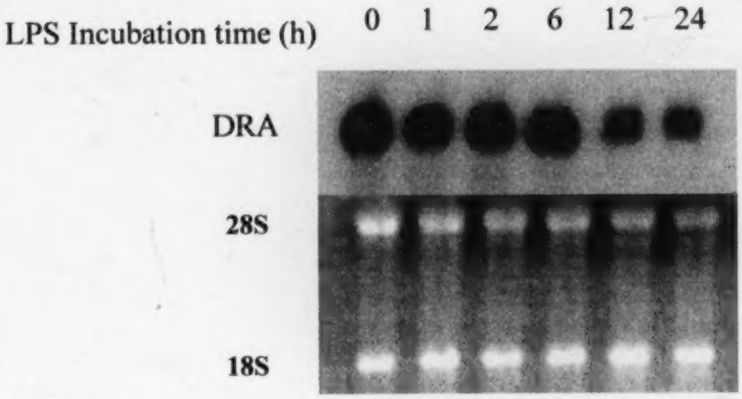

LPS Incubation time (h)
0
1
2
6
12
24
DRA
28S
18S

Figure 3-10. Northern blot analysis of MHC class II DRα expression of duck macrophages at various time points after exposure to LPS (1μg/mL).

Gene name	Primer	Primer sequence 5' to 3'
DRA	DRαF1	GCCGTCCGTCATCACCATCACCT
	DRαF2	AGCCGGACCCTGTGCCCCTTCA
	DRαF3	AACCGCTCGCAGGGCACCATCGC
	DRαR1	TGCCTGGCGGTAGAAGACGGT
	DRαR2	GCCACCCCGGAGCAGCCTGAG
	DRαR3	TCCCAGTGCGTCATCAGCGGCTC
Ii	IiF1	CCCCCTCCCTAGACGACACACC
	IiF2	CCGCAGCAGATCCCCAGTCCCTT
	IiF3	CCTGCCTCTCGCCCCCTCCCTAG
	IiR1	TGCTGCTGATCCCCACCCTGA
	IiR2	GCGCCCAGCTTGAGCATGTCCAC

Table 3-1. Oligonucleotide primers for MHC class II DRA and Ii.

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Chapter 4

In vitro characterization of the adherent peripheral blood mononuclear cells of Pekin ducks, *Anas platyrhynchos*

4.1 Introduction

Pekin duck (*Anas platyrhynchos*) is an important preclinical animal model for the study of hepatitis B. Exploration of the duck immune responses to duck hepatitis B virus is limited by the lack of knowledge of the duck immune system. Macrophages play a key role in avian host defense involved in both innate and adaptive immune responses (1). Elucidation of the physical and functional properties of duck macrophages may advance our knowledge of the duck immune system, which is vital in understanding the duck immune responses to bacterial or viral infections. Macrophages are mononuclear phagocytic cells, known for their ability to engulf a large number of foreign substances, bacterial cells and cell debris. In response to certain pathogens, macrophages are capable of producing highly reactive nitrogen intermediates to destroy and eliminate microbes (2). In addition, macrophages are professional antigen-presenting cells (APC), responsible for processing and presenting antigens to lymphocytes to stimulate adaptive immune responses.

Antibodies to distinguish duck monocyte/macrophage from other blood cells are not yet available. Due to the high capacity of macrophages to adhere to the tissue culture plastic, duck peripheral blood mononuclear cells (PBMCs) separated from other cells by the adherence method were normally presumed to be macrophages by many studies (3-5). In this study, we investigated the presence of macrophages in the adherent PBMC culture by characterizing the physical and functional properties of the adherent PBMCs from ducks. Pekin duck cDNAs encoding duck MHC class II DRA and invariant chain homologues of the mammalian counterparts have been identified in our lab. These sequences served as useful markers for identifying antigen-presenting cells in ducks.

Polymeric particles composed of poly(D,L-lactic-glycolic acid) (PLGA) were used in this study to investigate the phagocytic ability of the adherent PBMCs from ducks. PLGA particles were under intense investigations for their potential as an antigen delivery system for vaccines (6). Antigens delivered by PLGA particles have been shown to target and facilitate antigen delivery to antigen-presenting cells (APCs), enhance immune responses to the encapsulated antigen (7-9), and promote cross antigen presentation to CD8$^+$ cytotoxic T lymphocytes (CTLs) (10, 11). To evaluate the potential of PLGA nanoparticle as an antigen delivery system in ducks, the phagocytic ability of duck cells for PLGA particles was investigated in this study. Tetramethylrhodamine-labeled dextran (TMR), a macromolecule fluorescence probe was encapsulated in the PLGA particles and used as a tracer for the phagocytic process. The parameters in controlling the particle uptake such as the size of the particles, incubation time and the presence of immune stimulant LPS were also examined.

In the present study, we report that the adherent PBMC population contained duck cells with physical and functional properties characteristic of macrophages. The uptake of PLGA particles by the adherent PBMCs was limited by particle size. A cumulative increase in particle uptake was observed during 24-hour particle incubation with the adherent PBMCs. LPS treatment promoted the antimicrobial functional activities of the adherent PBMCs. Cells with ingested fluorescent particles were separated by a fluorescence-activated cell sorter (FACS). The cells expressed MHC class II DRA and invariant chain genes, hinting at the role they play as antigen-presenting cells in ducks.

4.2 Materials and Methods

4.2.1 Animal model

Pekin ducks were bred and housed at the Health Science Laboratory and Animal Care facility at University of Alberta (kindly provided by Dr. D.L. Tyrrell). The care and the use of laboratory animals complied with the Canadian Council on Animal Care (CCAC) guidelines.

4.2.2 Isolation of monocytes from duck PBMCs

Peripheral blood mononuclear cells (PBMCs) were separated on Ficoll-Paque Plus™ (specific gravity 1.077+0.001g/cm^3; Amersham Biosciences, Uppsala, Sweden) in accordance with the manufacturer's recommendations. The mononuclear cells were collected and washed twice with RPMI medium supplemented with 20% fetal calf serum, 2 mM L-glutamine and 100 U/mL penicillin and 100 μg/mL streptomycin and 1.0 mM sodium pyruvate (RPMI-20). The PBMCs (10-15 x 10^6 cells/mL) in 10 mL RPMI-20 were cultured in a 75-cm^2 tissue culture flask (Corning Incorporated-Life Sciences, Kennebunk, ME) at 37° C in 5% CO_2 incubator. After 24 h of cultivation, the non-adherent cells were removed with fresh medium added. The adherent PBMCs cultures were used after 2 to 3 days of cultivation. The final yield of the adherent PBMCs was approximately 0.5 % of the isolated PBMCs.

4.2.3 Morphological and cytochemical analysis of cultured monocytes

Duck macrophages were collected, counted by cell haemocytometer and placed onto a microscopic slide (Cytospin II, Shandon Instruments). The cytospin slides were stained with Wright-Giemsa stain (Fisher Scientific, Pittsburgh, PA) for microscopic analysis.

4.2.4 Characterization of PLGA particles

4.2.4.1 Preparation of PLGA particles (microparticles versus nanoparticles)

PLGA particles were prepared by the double solvent evaporation method as described earlier (12, 13). Poly(D,L-lactic-*co*-glycolic acid) (PLGA) polymer with inherent viscosity of 0.7 dL/g, composition 50:50 and average molecular weight 60,000 g/mol was purchased from Birmingham Polymers, Inc., Birmingham, AL. A red fluorescent chromophore TMR with nominal molecular weight of 40,000 was obtained from Molecular Probes, Inc., Eugene, OR. Poly(vinyl alcohol) (PVA) (molecular weight 31,000-50,000 g/mol, 89% hydrolyzed) was obtained from Sigma-Aldrich Canada, Ltd., Oakville, ON, Canada. Chloroform (HPLC grade) was purchased from Fisher Scientific International, Inc., Nepean, ON, Canada. Briefly, a solution of (25 mg/mL w/v; 100 μL) TMR-labeled dextran was emulsified in a solution of PLGA in chloroform (100 mg in 400 μL) by sonication at level 4 for 15 seconds using a microtip sonicator. (Model XL 2010; Heat Systems, Inc., Farmingdale, NY). This primary emulsion was then further emulsified in 2 mL 9% w/v polyvinyl alcohol (PVA) in phosphate buffered solution (PBS) with sonication at level 4 for 20 seconds to form a secondary emulsion. The resulting double emulsion was then added drop-wise to a stirring solution of PVA/PBS (9% (w/v); 8 mL). The emulsion was left to stir at room temperature for 3 hours to allow the evaporation of chloroform. The resulting particles were washed three times with sterile PBS (4 °C) and frozen immediately in a dry ice/acetone bath. They were then freeze-dried under vacuum for 3 days. Large PLGA microparticles were prepared similar to the method described above except during the emulsification procedures, the samples were mixed by vortex rather than sonication.

4.2.4.2 Estimation of TMR-labeled dextran loading in particles

The amount of TMR loaded in the PLGA particles was determined by measuring their UV absorbance at 555 nm. First, TMR was eluted from PLGA particles (2 mg) by stirring in 1 mL 5% SDS/0.5 M NaOH v/v overnight. The hydrolyzed samples were centrifuged and the supernatant containing the fluorescent probe was collected and neutralized to pH 7 with 1 M HCl before analysis. We prepared a series of standards of known concentration of TMR. The absorbance assay was measured in a 96-microwell plate (Gibco BRL) at 555 nm by a scanning spectrophotometer (PowerWave X; Bio-Tek Instruments, Inc., Winooski, VT). Data analysis was performed by KCjunior software. The percent loading of TMR was calculated by comparing the UV absorbance of the unknown with a standard curve.

4.2.4.3 Measurement of particle sizes

The mean volume diameter of the nanoparticles was determined by photon correlation spectroscopy (PCS) (Zetasizer 1000HS; Malvern Instruments, Ltd., Cirencester, UK) at 633 nm. 100 μL of PLGA nanoparticles (2 mg/mL w/v) was dispersed in 10 mM NaCl in a cuvette for size measurements. CONTIN analysis was performed to measure the size and distribution of the particles.

4.2.5 *In vitro* phagocytosis

4.2.5.1 Scanning Electron Microscopy (SEM)

The morphology and the dynamics of particle uptake by the duck macrophages were examined by scanning electron microscope. Duck PBMCs were allowed to adhere on sterile microscope slides placed in cell culture dishes. PLGA nanoparticles in RPMI-20 (2 mg/mL (w/v); 100 μL) were added to cell cultures (10 mL). After six hours of incubation, free particles were removed from duck adherent PBMCs. The cells were then fixed with 2.5% cacodylate buffer (pH 7.2) at room temperature for 1 hour, coated with gold (30mA for 5 min) and subjected to electron microscopy (Hitachi S-2500; Hitachi, Ltd., Tokyo, Japan).

The surface morphology of the PLGA microparticles and PLGA nanoparticles were examined using SEM. PLGA particles suspended in PBS were placed on metal stubs. The samples were placed in a sputter coater (S150B; BOC Edwards, Sussex, UK) for 40 s to produce a gold coating of approximately 30 nm in thickness. The coated samples were viewed under a scanning electron microscope.

4.2.5.2 Transmission Electron Microscopy (TEM)

The ultrastructure of the duck macrophages and intracellular localization of the PLGA nanoparticles within duck cells were examined under TEM. Duck adherent PBMCs were collected by centrifugation at 1,000 rpm for 5 min. The cell pellets were fixed with 2.5% cacodylate buffer (pH 7.2) as a primary fixative at room temperature for 1 hour. They were then post fixed in 1% osmium tetraoxide, dehydrated in ethyl alcohol, and embedded in Spurr's medium. Ultrathin sections were prepared and the samples were stained with uranyl acetate.

4.2.5.3 Confocal Laser Scanning Microscopy (CSLM)

To visualize the kinetics of intracellular uptake of the TMR-nanoparticles, cell samples were collected, fixed at designated time intervals and viewed under a confocal laser scanning microscope (Zeiss 510 LSM NLO; Carl Zeiss Microscope Systems, Jena, Germany). Briefly, PBMCs (2 x 10^6) were placed in cell chamber wells (Lab-Tek® Chamber Slide™ System, Nalge Nunc International, Naperville, IL) containing 300 μl RPMI-20. Cell samples were incubated at 37 °C and 5% CO_2 for 24 h before removing the non-adherent cells. The duck adherent PBMCs were cultured for two more days before analysis. LPS was added at a final concentration of 500 ng/mL (w/v). PLGA particles (nanoparticles or microparticles) in RPMI-20 (2 mg/mL; 40 μL) were added to appropriate wells. At designated time intervals, free PLGA particles were removed by washing the wells with PBS buffer (pH 7.32). The cell membranes were stained with a green fluorescent chromophore Alexa Fluor 488 conjugated to concanavalin A (Molecular Probes, Inc., Eugene, OR) in PBS (100 μl; 0.0005% (w/v)) for 2 minutes. The excess membrane-labeling fluorescent probe was removed by washing the cells five to six times with PBS. The cell samples were then fixed with 100 μL of 4% w/v paraformaldehyde in PBS pH 7.4 for 10 minutes. A solution of mounting medium (100 μL) containing 10% w/v Mowiol 488 (Fluka, Sigma-Aldrich Canada, Ltd., Oakville, ON, Canada); 2.5% w/v 1,4-diazabicyclo(2.2.2)octane (DABCO) (Aldrich Chemical Company. Inc., Milwaukee, WI) and 25% v/v glycerol in 0.2M Tris-buffer pH 8.5 was prepared and added to each wells. The slides were analyzed under a confocal laser scanning microscope (CSLM) with argon laser (488nm) and helium neon laser (543nm) using a 63X objective under oil immersion. Images were digitally recorded in a 512x512 pixel image size.

4.2.5.4 Fluorescence-activated cell sorter (FACS) analysis and isolation of phagocytic cells

Duck adherent PBMCs were prepared as described above. PLGA nanoparticles (2 mg/mL (w/v); 400 μL RPMI-20) were added to the adherent PBMC cultures. Phagocytosis inhibitor cytochalasin B (Sigma-Aldrich Canada, Ltd., Oakville, ON, Canada) (5 μg/mL (w/v)) was pre-incubated with the adherent PBMCs thirty minutes prior to the addition of fluorescent particles. Low temperature samples were placed in the fridge at 4 ° C immediately after adding the nanoparticles. LPS (*E. coli* 026.B6) obtained from Sigma, Canada was added to the LPS stimulated cells at a final concentration of 500 ng/mL w/v. After incubating with the particles for twenty-four hours, the duck adherent PBMCs were washed five times with RPMI-20 to remove free particles. Subsequently cell samples were gently removed using a cell scraper (Corning Incorporated, Corning, NY) and suspended in a cold FACS buffer (PBS supplemented with 2mM EDTA, 1% v/v FCS and 0.05% w/v NaN_3). The cell suspension was then transferred to a BD Falcon® tube and processed by a FACScan® flow cytometer (Becton Dickinson) to detect fluorescence emission.

Flow cytometric analysis was performed using the Cellquest software (Becton Dickinson). Analysis was conducted on 10,000 events from the cell suspension. Data were acquired on live cells excluding cell debris and uningested free particles, which was determined by their pattern on the forward light scatter (FSC) versus side light scatter (SSC). Duck cells having ingested TMR-PLGA nanoparticles were expected to display red fluorescence activity (FL-2) above background signal. Detector gain value for collecting signals from TMR was fixed throughout the study. The mean fluorescence intensity (MFI) is a measurement of the average fluorescence intensity above the background. The fluorescence intensity of duck macrophages before phagocytosing TMR-PLGA nanoparticles was set as the background fluorescence. Cell samples that displayed fluorescence signals above background were sorted by FACScaliber cell sorter (Becton Dickinson) and collected for RNA isolation.

4.2.6 Oxidative burst

4.2.6.1 Greiss assay

Nitric oxide production was measured by determining the nitrite levels in the culture supernatants using the Greiss assay. A series of LPS concentrations was added to the designated wells containing duck macrophages. N^G-monomethyl-L-arginine (L-NMA) purchased from Calbiochem, EMD Biosciences, In., La Jolla, CA, (250 μM) was used as a competitive inhibitor of nitric oxide synthase to confirm the role of nitric oxide as an effector molecule. Duck adherent PBMC samples were incubated at 37 °C. At predetermined time intervals, 50 μl of supernatant was collected and transferred to a 96-microwell plate. The samples were stored at 4 °C until all supernatant samples were collected and analyzed using a colorimetric assay with Greiss reagent (14). Briefly, an equal volume of Greiss reagent (one part of 1% w/v sulfanilamide and one part of 0.1% w/v naphthylethylenediamine dihydrochloride) was added to the wells. After 10 minutes of incubation at room temperature, the absorbance at 555 nm was measured in a 96-microwell plate (Gibco BRL) by a scanning spectrophotometer (PowerWave X; Bio-Tek Instruments, Inc., Winooski, VT). Data analysis was performed by KCjunior software. All samples were prepared in triplicate.

4.2.7 MHC class II DRA and invariant chain expression by phagocytic cells

RNA samples were isolated from the particle-ingested duck cells by Qiagen RNeasy Mini Kit (Qiagen, Inc., Mississauga, ON, Canada). The first strand cDNA was synthesized from total RNA using an Oligo (dT)20 primer. Five micrograms of total RNA was used as template, and incubated with ThermoScript™ RNase H^- reverse transcriptase at 50°C for 45 minutes, as directed by the manufacturer protocol (Invitrogen). Specific oligonucleotide primers were designed against MHC class IIα and invariant chain sequences. The forward and reverse oligonucleotide primer sequences used in the PCR for MHC class IIα were 5'-AACCGCTCGCAGGGCACCATCGC-3'and 5'-

TCCCAGTGCGTCATCAGCGGCTC-3', respectively. The forward and reverse oligonucleotide primer sequences for the invariant chain were 5'-CCTGCCTCTCGCCCCCTCCCTAG-3', and 5'-GCGCCCAGCTTGAGCATGTCCAC-3', respectively. The PCR assay was carried out in the following conditions: 25 successive cycles of denaturation at 94 °C for 30 sec, primer annealing at 63 °C for 30 sec, and DNA extension at 72 °C for 1 min.

4.3 Results

4.3.1 Particle characterization

Two different size ranges of fluorescent-probe-loaded PLGA particles were produced using different amount of energy during the emulsion formation process (vortex versus sonication). The average size of the PLGA microparticles containing TMR (TMR-microparticles) was approximately 18 μm in diameter (ranged between 14 to 20 μm) estimated by scanning electron microscopy (Figure 4-1B). Measured by photon correlation spectroscope, the mean volume diameter of the PLGA nanoparticles containing TMR (TMR-nanoparticles) was 658 nm (polydispersity: 0.67). Both the micro and nanoparticles (Figure 4-1) appeared to be spherical in shape and had an essentially smooth surface under the scanning electron microscope. Occasionally, a few cavities on the surface of both micro and nanoparticles were observed. The TMR loading of the PLGA nanoparticles was 0.266 % w/w per dry weight of nanoparticles, whereas the TMR loading for PLGA microparticles was 0.100 % w/w per dry weight of the nanoparticles. The fluorescence activities of both of the nanoparticles and the microparticles were adequate for study the intracellular uptake as they both gave strong fluorescence signals when viewed under the confocal laser scanning microscope.

4.3.2 Morphological and cytochemical analysis of duck adherent PBMCs

Duck adherent PBMCs contained the distinctive features of avian macrophages (15). Their cell surfaces were irregular in shape with numerous pseudopod-like projections. Under light microscope, the nuclei varied from round to bilobed kidney shape. The cytoplasm of macrophages stained blue-grey with Wright-Giemsa stain and contained several visible vacuoles (Figure 4-2). A light area and a more deeply stained area could be observed as two distinct zones in the cytoplasm.

4.3.3 Uptake of PLGA particles by duck macrophages

4.3.3.1 Electron microscopic analysis

To evaluate the phagocytic ability of the adherent PBMCs, the cultured duck adherent PBMCs were incubated with TMR-nanoparticles and examined under scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Scanning electron microscopic images shown in Figure 4-3 (A-C) demonstrated the dynamics of the phagocytosis process. Some nanoparticles were bound on the surface of the cell membrane while others were engulfed by the cells and appeared as a half-dome or spherical shape under the cell membranes. Transmission electron microscope revealed that PLGA nanoparticles taken up by duck cells were remained in the phagosomes inside the cells (Figure 4-4 (B-C)). PLGA nanoparticles appeared as dark and round spheres under the electron microscope.

4.3.3.2 Flow cytometric analysis

To identify and select the cell population that have ingested TMR-nanoparticles, duck cells were analyzed by flow cytometry before and after they internalized the TMR-nanoparticles. The duck adherent PBMCs appeared as a single population in region A on the dot plot before incubation with TMR-nanoparticles (Figure 4-5 A). Having ingested the TMR-nanoparticles, duck cells were shown to increase in size and granularity as evidenced by the increased number of cells localized at a relatively higher forward and side scatter within region A (Figure 4- 5 B). Duck cells located in this region were also included during analysis of fluorescent activity. The TMR-nanoparticles appeared as a streak on dot plot, which is close to the size ranges of cell debris and dust (Figure 4-5 C). They were excluded in the analysis of fluorescent activity for the cells with ingested particles.

The fluorescence intensity of duck macrophages before phagocytosing TMR-nanoparticles represented background fluorescence. It was set below 1x 10^1 as the threshold intensity on the FL-2 scale for "red" fluorescence (Figure 4-5 D). Cells exhibiting fluorescence above this baseline threshold were considered positive for TMR-nanoparticle-ingested cells. Co-culturing of the adherent PBMCs and TMR-nanoparticles for 24 h resulted in two peaks on a histogram (Figure 4-5 E). The peak on the left had a similar fluorescence intensity range as the background fluorescence of duck macrophages, indicating that some of the PBMCs did not take up TMR-nanoparticles. The peak on the right with fluorescence intensity above background represented the cell population that had internalized TMR-nanoparticles. Based on these criteria, duck cells that ingested TMR-nanoparticles were selected and isolated by FACScaliber cell sorter.

4.3.4 Kinetics of TMR-PLGA nanoparticle uptake

To determine the kinetics of TMR nanoparticle uptake by duck PBMCs over a 24-h period, cell samples incubated with TMR-nanoparticles were examined under confocal laser scanning microscope over 2, 4, 6, 8 and 24 h time intervals. Confocal laser scanning microscopic images showed minimal phagocytosis of TMR-nanoparticles had taken place in the first two hours of incubation, as only a few cells contained intracellular red fluorescence particles (Figure 4-6 A). A steady increase in the frequency of cells containing TMR-nanoparticles, and a progressive increase in the number of internalized particles were observed over 4, 6, 8 and 24 h, as evidenced by an increase of punctate red fluorescence within the cells (Figure 4-6 (B-E)). Overall, the amount of nanoparticles ingested by adherent PBMCs increased with time for the first 24 hours of incubation.

4.3.5 Factors affecting the cellular uptake of PLGA particles: LPS, particle size, phagocytosis inhibitor and temperature

To assess the effect of LPS on the nanoparticle uptake over time, adherent PBMCs incubated with TMR nanoparticles were monitored at 2, 4, 6, 8 and 24 h time intervals using confocal laser scanning microscope (Figure 4-6 (F-J)). In the first two hours, the addition of LPS did not affect the uptake of nanoparticle. However, at 4, 6, 8 and 24 h, the number of TMR nanoparticles ingested by the duck cells and the cell fractions containing TMR microparticles were apparently higher compared to the cell cultures containing no LPS at the corresponding time points.

To determine the effect of PLGA particle size on their cellular uptake, TMR microparticles with a mean diameter of 18 μm were prepared, incubated with the adherent PBMCs culture for 24 h and observed under the confocal laser scanning microscope. TMR microparticles were not taken up the adherent PBMCs (Figure 4-6 K).

To quantify the effect of LPS on particle uptake by the adherent PBMCs, FACS analyses were performed on the adherent PBMCs, which were incubated with PLGA nanoparticles containing tetramethylrhodamine-dextran for 24 h in the presence or absence of LPS (Figure 4-7). One-colour flow cytometry was used to determine the percentage of cells positive for tetramethylrhodamine red fluorescence. FL-2 scale indicates red fluorescence intensity. Cells above the threshold 1 x 10^1 on the FL-2 scale were considered to be positive for red fluorescence. The percentage of cells positive for red fluorescence represents the proportion of duck cells that had internalized particles. The mean fluorescence intensity (MFI) represents the average amount of fluorescence of the positive cells. There were 54 % of positive cells with a MFI of 24 when the adherent PBMCs were cultured with TMR-PLGA nanoparticles. In the presence of LPS, approximately 66 % of positive cells with a MFI was 27, suggesting LPS treatment led to a slight increase in phagocytosis by the adherent PBMCs. The enhanced phagocytic activities induced by LPS corroborated with the findings in the CLSM studies (Figure 4-6 (F-J)) confirming the stimulatory effect of LPS on the phagocytic capacity of the adherent PBMCs.

To assess the mechanism of the nanoparticle uptake, the adherent PBMCs were incubated with TMR-nanoparticles in the presence of cytochalasin B or at low temperature (Figure 4-7). Phagocytosis was completely suppressed by incubating the cells with TMR-nanoparticles at 4 °C. The uptake of TMR-nanoparticles was also aborted in the presence of cytochalasin B, an agent inhibiting phagocytosis but not pinocytosis (16). Since low temperature or the presence of cytochalasin B prevented particle uptake, it was concluded that the uptake was a result of particle phagocytosis and not because of pinocytosis of free fluorescence dye, or superficial fluorescence particle and cell association.

4.3.6. MHC class II DRA and invariant chain expression by phagocytic duck cells

To characterize the duck phagocytes, RNA was isolated from the cells that ingested particles selected by fluorescence-activated cell sorter. RT-PCR was performed for gene expression analysis using MHC class II DRA and invariant chain sequence-specific primers. The particle-ingested cells expressed MHC class II DRA and invariant chain genes. Exposure to LPS during the 24 h particle incubation period did not affect the expression of MHC class II DRA and invariant chain in the phagocytic cell population (Figure 4-8).

4.3.7 Respiratory burst by the adherent PBMCs

To test the ability of the adherent PBMCs to produce nitric oxide in response to activation with LPS, reactive nitrogen intermediates were measured by sampling culture supernatants for the presence of nitrite (NO_2^-), using the Greiss reaction. Increased concentration of LPS resulted in the production of more nitrite by the adherent PBMCs (Figure 4-9). The presence of LPS resulted in an increased of nitrite production during the first 72 h incubation period (Figure 4-10). A low level of NO_2^- was produced in unstimulated cells. The production of

NO_2^- triggered by LPS was markedly inhibited by L-arginine analog (L-LMA), confirming the involvement of the nitrogen oxidative pathway (Figure 4-10).

4.4 Discussion

Macrophages are characterized by their high capacity to adhere to the tissue culture plastic. Duck macrophages described in the literature were purified from the blood cells by the adherence method (3-5). Approximately 83% of the adherent PBMCs from chicken were shown to be monocyte-derived macrophages after culturing for 48 hours (17). However, whether duck macrophages are present in the adherent PBMC culture has not been examined. Therefore, in this study we investigate the physical and functional properties of the adherent PBMCs and provide evidence for the presence of macrophage-like cells in the adherent PBMCs.

In avian blood, the monocyte cell number varied between 0.5%-5% of the white cell count (18). We found that about 0.5 % of the duck PBMCs remained adherent to the tissue culture plastic after 48-72 hours of incubation. At an ultra-structural level, most adherent PBMCs were similar to those reported for avian macrophages (1). Scanning electron microscopy of the duck cells having ingested particles revealed that they were large and round leukocytes, around 20 μm in diameter, irregular in shape with few pseudopodia present on the cell surface. Transmission electron microscopy confirmed the internalization of PLGA particles and illustrated the ingested PLGA particles were located within the phagosomes. These cells had an irregular nucleus, a moderate to large amount of cytoplasm with a variable number of vacuoles evident. Moreover, the cytochemical staining of the adherent PBMCs showed staining pattern characteristic of macrophages.

Phagocytosis is an important defense mechanism of innate immunity. Macrophages are specialized phagocytic cells that engulf and destroy invading microorganisms, cell debris and particles. In this study, we have shown that the internalization of the TMR-nanoparticles by the adherent duck cells was largely mediated by an active phagocytic process not pinocytosis. Approximately 30%

of the adherent PBMCs from ducks were phagocytic for TMR-nanoparticles. A similar percentage of phagocytic cells was found in the adherent PBMCs from chicken with about 20-30% of the adherent macrophages in chickens able to internalize sheep red blood cells (SRBC) (19). Macrophages are a heterogeneous group of cells with a diversity of phenotypes, differentiation and activated states, and functional abilities. The fact that only one third of the adherent PBMCs from ducks were phagocytic for TMR-nanoparticles suggested that there were a non-homogeneous and distinct macrophage populations present in the adherent PBMCs. Preliminary *in vitro* data suggests that saturation of PLGA nanoparticle uptake by duck cells were reached within 24 hours, which is similar to the kinetics of particle uptake by human dendritic cells and macrophages (20).

Macrophages destroy phagocytosed bacteria by concerted effects of the oxidative burst, acidification, lysosomal enzymes and antimicrobial peptides (21). Nitric oxide produced by chicken macrophages during respiratory burst exerted both antimicrobial activity and immunoregulatory effects (22, 23). Like chicken macrophages, the adherent PBMCs from ducks exhibited oxidative burst and produced NO when stimulated by LPS. Furthermore, the nitrite production by the duck cells was dependent on the time of LPS exposure and the concentration of LPS. The phagocytic capacity of the duck cells was also increased in the presence of LPS with a relatively higher number of fluorescence particles internalized by the cells during the first 24 hours incubation. These findings are consistent with the functional characteristic of avian macrophages (24, 25).

MHC class II molecules and invariant chain are both involved in processing and presenting antigens to CD4$^+$ T cells to initiate the adaptive immune response (26). Phagocytic duck cells isolated based on their ability to internalize TMR-nanoparticles expressed MHC class II DRA and invariant chain genes, which are consistent with the gene expression profile of macrophages acting as an antigen-presenting cell. Human and mouse studies showed that the surface expression of MHC class II was influenced by both transcriptional and posttranslational control (27-30). Transcription control of MHC class was primarily mediated by class II transactivator (CIITA) (27, 28). Moreover, MHC class II expression was also regulated by the stabilization of MHC class II mRNA and the MHC class II-protein complex on the cell surface (29, 30). Although MHC class II DRA gene expression by the particle-ingested cells was not altered by LPS, it is unclear whether the LPS effects on the MHC class II DRA transcript level correlated with the surface expression of MHC class II in duck cells.

While nanoparticles of an average of 700 nm in diameter were internalized by the adherent PBMCs, large PLGA microparticles greater than 14 μm were not. Thus the size of particles is a critical factor in determining the uptake by duck cells. The importance of the size of particles on phagocytosis is not new. Many *in vitro* and *in vivo* murine studies have shown that phagocytic uptake of particles is dependent on the size of the particles (31-33). In rat, fluoresceinamine bound PLGA particles with a mean diameter of 265 nm (ranging from 110 to 670 nm) were readily phagocytosed by macrophages in the synovium whereas particles with a mean diameter of 26.5 μm (ranging from 3.1 um to 59.9 μm) were not phagocytosed (34). PLGA particles as an antigen delivery system were designed to deliver antigens more effectively to APC for potentiating the immune response (35-37). A study had shown that small particles (1-10 μm) produced stronger immune responses than large particles (>10 μm), which was related to their uptake by the local macrophages and their delivery to the draining lymph nodes (38). Therefore, the size of the particles appears to correlate with the magnitude of the induced immune response. This study demonstrated the ability of duck macrophages to internalize PLGA nanoparticles suggesting that it is possible to use PLGA nanoparticles to deliver antigen to duck antigen-presenting cells, and to enhance immune responses of the incorporated antigens in ducks. *In vivo* studies are needed to determine the optimal size of the PLGA particles for uptake and the effects of antigens delivered by PLGA particles on the immune responses in the duck system.

Overall, our data indicate that macrophages are present in the primary duck cell culture from the adherent PBMCs. The adherent PBMCs from ducks displayed morphological, cytochemical features and functional activities that are characteristic for avian macrophages (1, 15). The adherent PBMCs from duck were capable of performing antimicrobial activities through phagocytosis and NO production. The duck cells having ingested particles expressed MHC class II DRA and invariant chain, suggesting that these cells are antigen presenting cells and likely to be duck macrophages. PLGA nanoparticle were taken up by duck cells while large PLGA microparticles were not. This is particularly relevant to the design of vaccine formulations using polymeric particles. To the best of our knowledge, this is the first study to report the ability of duck cells to phagocytose polymeric PLGA particles. The ability of duck cells to internalize PLGA nanoparticle suggests that PLGA nanoparticles may be used as an approach to stimulate and augment immune responses in ducks. Further studies on characterizing the immune responses to antigen delivered by PLGA nanoparticles in ducks are warranted.

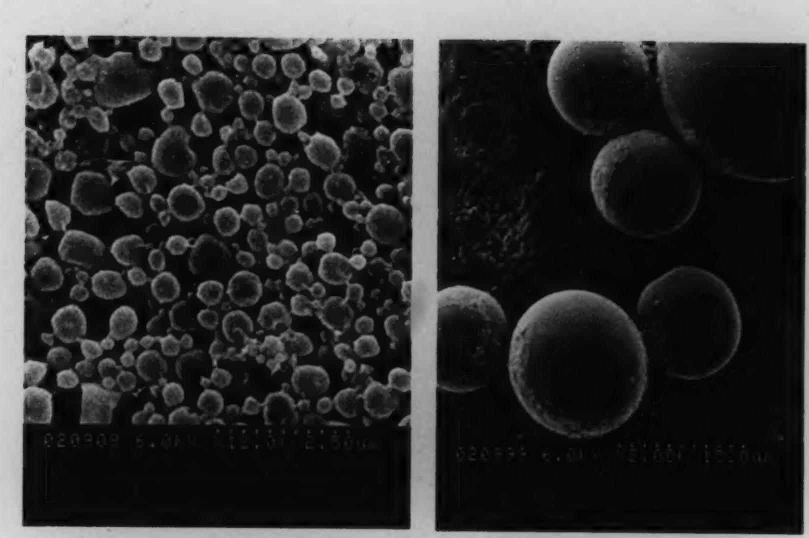

Figure 4-1. Scanning electron micrographs of PLGA nanoparticles (A) and PLGA microparticles (B).

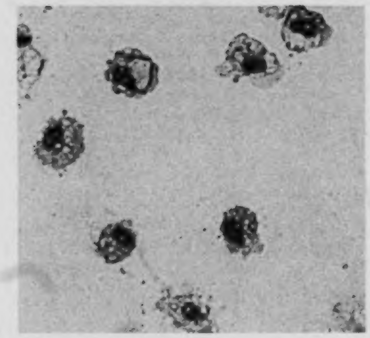

Figure 4-2. Light microscopic image of duck macrophages derived from blood monocytes x 63 under differential interference contrast (DIC) oil-immersion objective.

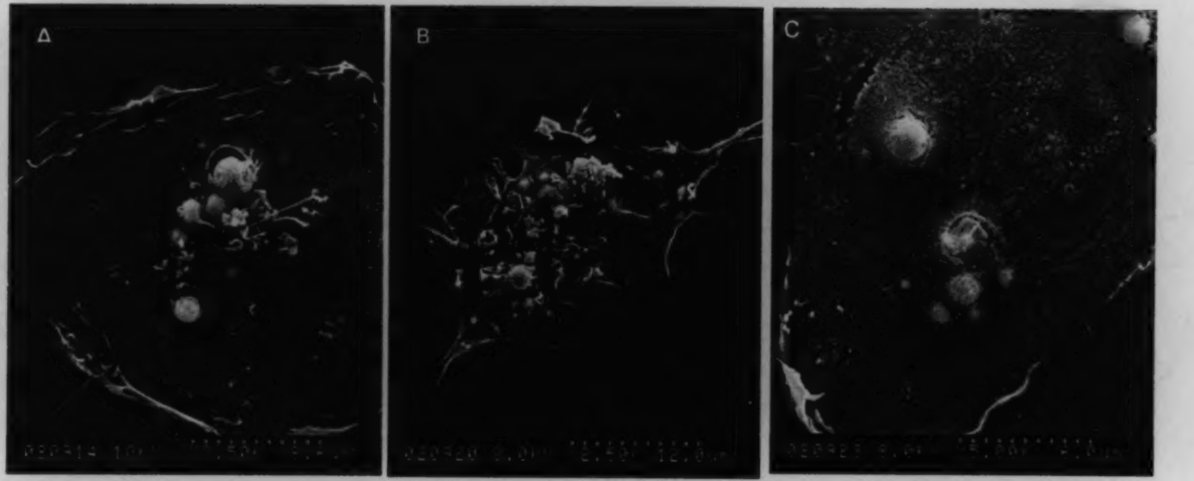

Figure 4-3 (A-C). Scanning electron micrograph of duck macrophages internalizing PLGA nanoparticles. Duck macrophages were fixed after an incubation of 6 h with PLGA nanoparticles.

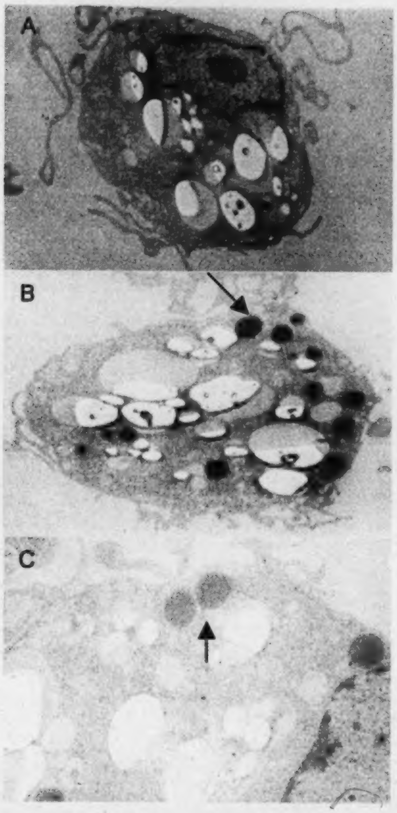

Figure 4-4. Transmission electron micrographs of duck macrophages derived from blood monocytes. (A) Duck macrophages x 2400; (B) Duck macrophages that had internalized PLGA nanoparticles x 2400. (C) Higher magnification of duck macrophages containing PLGA nanoparticles x 3600. Arrows indicated PLGA nanoparticles.

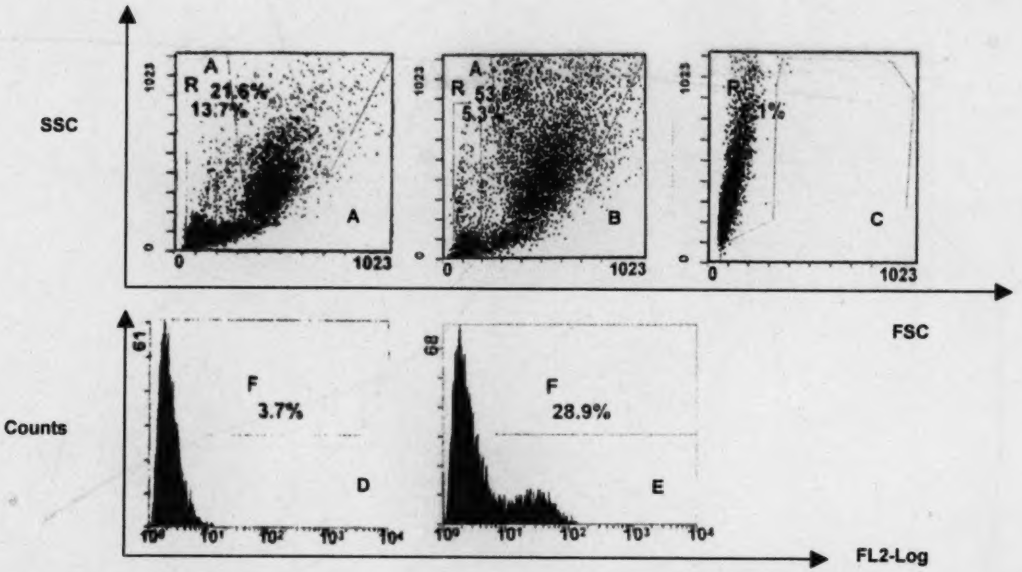

Figure 4-5. (A) Forward and side scatter dot plot (FSC/SSC) of duck macrophages derived from blood monocyte. (B) Dot plot (FSC/SSC) diagram of macrophages that have ingested TMR-PLGA nanoparticles. (C) Dot blot representing the TMR-PLGA nanoparticles. (D) Histogram of 10^4 events showing the fluorescence background from duck macrophages (No gate). (E) Histogram showing the overall fluorescent activity of macrophages after incubation with TMR-PLGA nanoparticles (No gate). The peak with higher fluorescence intensity (FL-2) represents the macrophage cell population that have ingested TMR-PLGA nanoparticles.

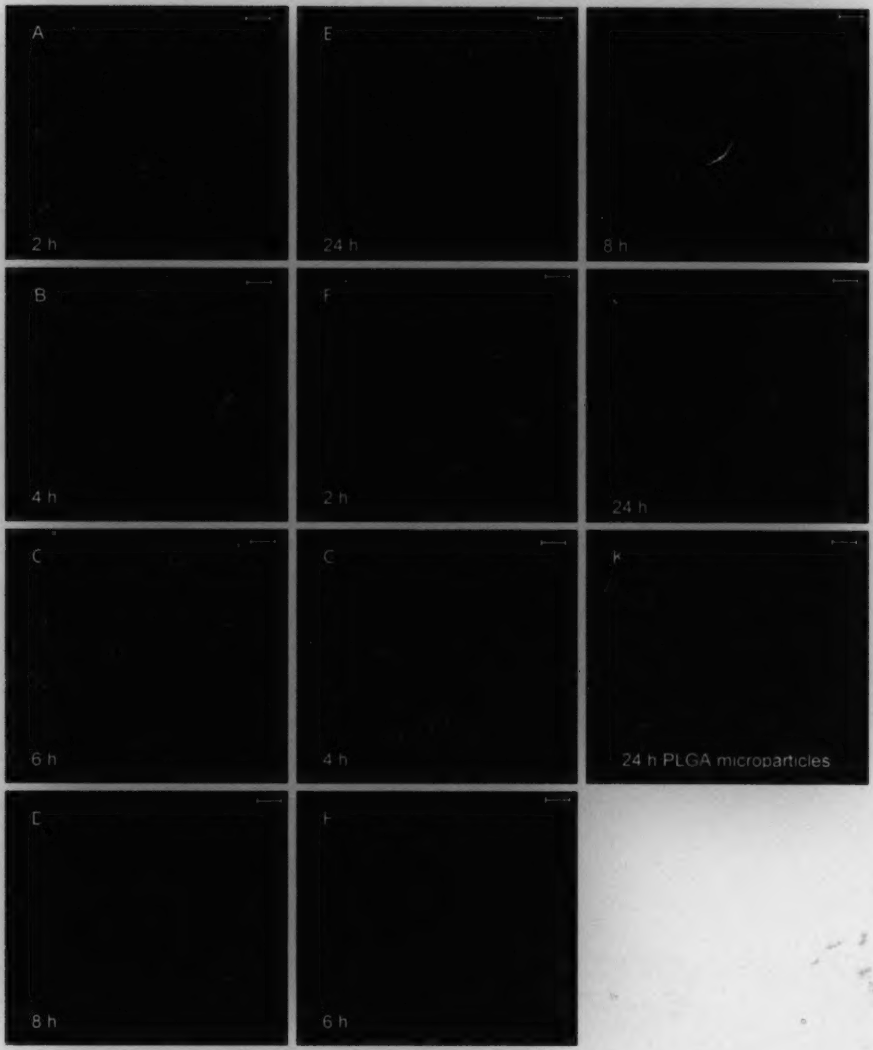
A
2 h
E
24 h
8 h
B
4 h
F
2 h
24 h
C
6 h
G
4 h
K
24 h PLGA microparticles
D
8 h
H
6 h

Figure 4-6. Confocal laser scanning micrographs of duck macrophages incubated with TMR-loaded PLGA nanoparticles over time in the absence of LPS (A-E) and in the presence of LPS (F-J). Image of duck cells after 24 hours incubation with large PLGA microparticles (K).

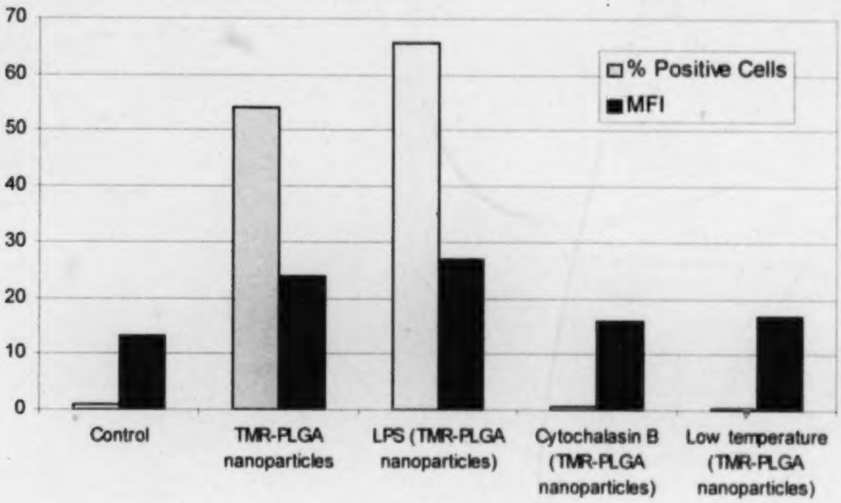

Figure 4-7. Flow cytometric analysis of the phagocytic response of duck macrophages to LPS, cytochalasin B or at low temperature. Duck macrophages were incubated with TMR-PLGA nanoparticles for 24 h in the presence or absence of LPS, cytochalasin B or at 4 °C.

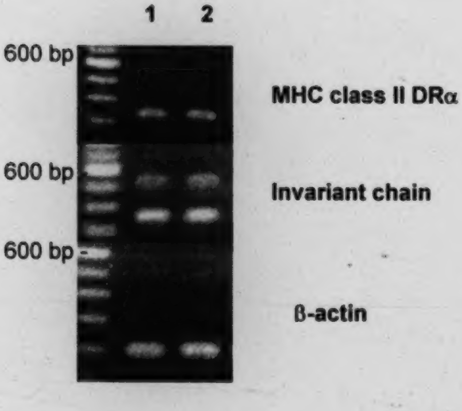
1
2
600 bp
600 bp
600 bp
MHC class II DRα
Invariant chain
ß-actin

Figure 4-8. Expression of MHC class II DRα and invariant chain were determined from the isolated phagocytic cells. Lane 1: Phagocytic cells; Lane 2: LPS-treated phagocytic cells. The β-actin served as a loading control.

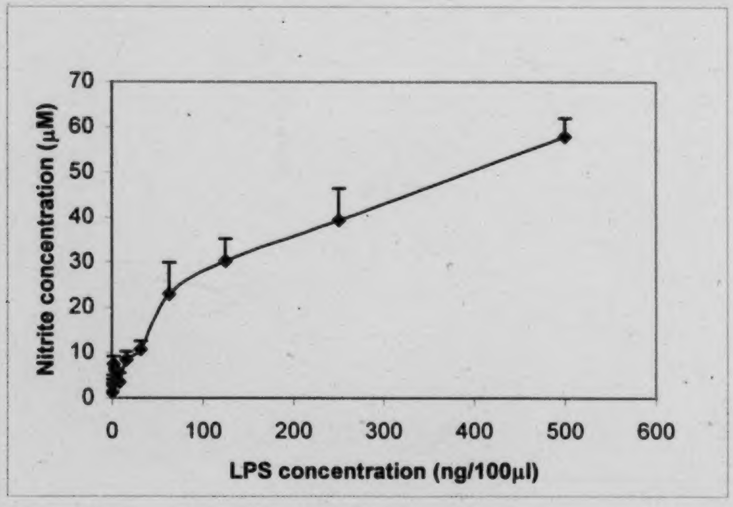

Figure 4-9. Effects of LPS on triggering nitrite (NO_2^-) by duck macrophages. After 48 hours incubation, 50 μL of culture fluid was removed and assessed for NO_2^- concentration.

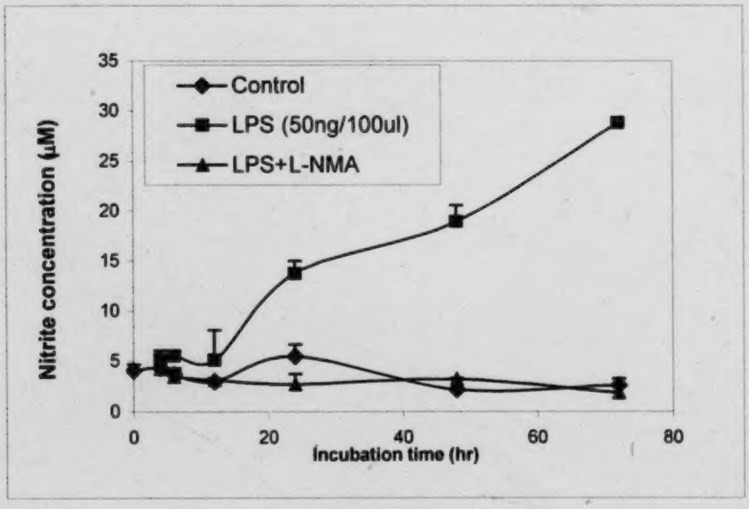

Figure 4-10. Time course of nitrite (NO_2^-) production by activated duck macrophages. At zero time point, 500 ng/mL of LPS and 250 µM N^G-monomethyl-L-arginine (LPS + L-NMA) were added in the appropriate wells. At indicated incubation times, 50 µL of culture fluids were colleted and assessed for NO_2^- concentration.

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Chapter 5

PLGA Nanoparticle Delivery of DHBV Vaccine in Pekin Ducks

5.1 Introduction

Hepatitis B virus (HBV), duck hepatitis B virus (DHBV), woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV), all belong to the same *hepadnaviridae* family. All of these viruses can cause both acute and persistent infections in their respective hosts. They have similar genomic organization, antigen composition and replication strategies (1). Among them, DHBV-infected ducks provide one of the most convenient and economical animal models for the study of human HBV infection (2). Since hepadnavirus replication is noncytolytic, the pathogenesis and the elimination of virus are generally accepted to be mediated by the host immune response. Age-related immune competency was associated with the transient or chronic infection outcomes in the DHBV experimental inoculation of ducks (3-5). Ducklings experimentally infected with DHBV within the first several days following hatching developed persistent viremia (viral load in blood). In contrast, adolescent or older ducks inoculated with DHBV developed mild hepatitis, but the infection was rapidly cleared from these ducks with the development of a neutralizing anti-DHBs antibody response (6). A large inoculum capable of overwhelming the duck immune system was another important determinant for the development of persistence infection (5, 7). Persistence infection in chronically infected DHBV-infected ducks resulted from poor immune responses. Viral clearance in DHBV-infected ducks appears to be dependent on the strength of the host's immune responses against the virus (8). Duck IFN-γ was shown to inhibit DHBV viral replication and induce viral cccDNA clearance in infected primary duck hepatocytes (9). The mechanism for viral clearance in DHBV infection appears to be similar to that in human HBV infection. The DHBV model is a useful system for the study of prophylactic and therapeutic vaccines.

Biodegradable PLGA nanoparticles have emerged as a promising vaccine delivery system to increase immune responses. PLGA nanoparticles enhance immune responses by targeting professional APC and delivering a high concentration of antigen directly to the APC (10-13), and by its ability to prolong antigen presentation by APC (14). A recent study showed that particulate antigen enhanced antigen presenting efficiency by altering intracellular trafficking of the antigen (15). Antigen delivered via PLGA particles induced strong cytotoxic T lymphocyte (CTL) (16-18) and Th1 CD4$^+$ immune responses (19) by the ability of the particle to deliver antigen effectively on MHC class I for cross-presentation to CD8$^+$ T lymphocytes as well as MHC class II for the induction of CD4$^+$ T lymphocytes (20, 21). CD8$^+$ T lymphocytes play an important role in elimination of viruses. Utilizing PLGA nanoparticles as a vehicle to deliver viral antigen as a therapeutic vaccine is a good strategy to induce CTL and Th1 CD4$^+$ T cell response against the virus.

In addition to "signal 1" (antigen recognition) provided by the peptide-MHC complex, a "signal 2" is required for the induction of the immune response. A "infectious non-self" model proposed by Janeway suggests that the recognition of the pathogen-associated molecular patterns (PAMPs) on bacteria by APC upregulates co-stimulatory molecules thus providing "signal 2" (22). This recognition process is mediated by a set of pattern recognition receptors (PRRs), now known as toll-like receptors (TLRs) (23). Signalling via TLRs has been shown to activate and trigger dendritic cell maturation which results in up-regulation of co-stimulatory molecules, cytokine secretion and migration of dendritic cells to secondary lymphoid organs to stimulate T cell responses (24). Therefore, an ideal HBV therapeutic vaccine should contain suitable adjuvants such as TLR agonists to activate antigen-presenting cells.

Monophospholipid A (MPLA) is an immunomodulator and an adjuvant (25) derived from lipopolysaccharide (LPS). It is devoid of the toxicity while retaining the adjuvant effect of LPS (25). MPLA used as an adjuvant has been shown to be safe and well-tolerated in human use (26). It has been demonstrated that the adjuvant properties of MPLA were mediated by signalling through TLR-2 and TLR-4 (27). In addition to activating dentritic cells to secrete IL-12, it also up-regulates CD40L expression on the T cell to promote and support optimal cellular immune activation (28). A previous study in the murine model had shown co-delivery of hepatitis B core antigen and an immunomodulator monophospholipid A by PLGA nanoparticles is promising for the induction of antigen-specific Th1 response (29). Therefore, it was hypothesized that the co-delivery of DHBcAg and MPLA by PLGA nanoparticles may induce effective anti-DHBV immune responses to facilitate viral elimination in ducks with chronic DHBV. The result of the study showed that DHBcAg along with MPLA formulated in PLGA nanoparticles as a vaccine were capable of eliciting antibodies against the DHBcAg in normal ducks. Vaccination of ducks with chronic DHBV also resulted in the generation of anti-DHBc IgY antibody. However, the virological outcomes (i.e. viral load in the blood) after immunization with the nanoparticle formulation were not significantly different from the control in the chronically DHBV-infected ducks.

5.2 Material and Methods

5.2.1 Animals

The Pekin ducks (*Anas platyrhynchos*) were bred and housed at the Health Science Laboratory and Animal Care facility at University of Alberta (kindly provided by Dr. D.L. Tyrrell). The care and the use of laboratory animals complied with the Canadian Council on Animal Care (CCAC) guidelines.

5.2.2 DHBV infection

Three different duck models were used in this study: normal uninfected Pekin ducks and two chronic DHBV infection models (experimentally infected and vertically transmitted). The experimentally infected group was generated by inoculating newly hatched ducklings with a 50-μl intravenous injection of serum containing Alberta strain of DHBV-16 (GenBank accession number AF047045). The virus inoculum used in the experiments was derived from pooled serum collected from ducks congenitally infected with Alberta strain of DHBV-16 containing $\geq 10^9$ DHBV genomes/mL. This model mimics the horizontal transmission of the HBV infection in man. The DHBV (Alberta strain of DHBV-16) congenitally infected ducks were used as the second type of chronic infection model. This model resembles the mother-to-infant vertical transmission in humans. Viremia was confirmed by dot-blot hybridization. Sera containing ≥ 10^8 DHBV genomes/ mL were considered DHBV positive.

5.2.3 Vaccination Protocol

Within each duck group, they were further divided into three subgroups to receive different vaccines: group A, PLGA nanoparticles carrying (DHBcAg+MPLA) vaccine, group B, DHBcAg alone and group C, PLGA nanoparticles alone. Blood samples were collected from the normal, DHBV congenitally infected and DHBV experimentally infected Pekin ducks, all between 2-3 months of age, one week prior the beginning of the immunization study. This was considered the zero time point. The vaccines were injected subcutaneously on the posterior side of the duck at the base of the neck. Each duck received three immunizations of the same type of vaccine at 2-week intervals. Sera samples were collected one week after each immunization. For the group receiving (DHBcAg+MPLA)-loaded PLGA nanoparticles, each vaccine dose contains 10 mg of nanoparticles comprised of 15 μg of DHBcAg and an estimate of 9 μg of MPLA. DHBcAg was given as a 15 μg dose per immunization. The control group received 10 mg PLGA nanoparticles in each vaccination. All vaccines were reconstituted in 200 μl sterile phosphate buffered saline (PBS).

5.2.4 Preparation of DHBcAg-loaded PLGA nanoparticles

Poly(D,L-lactic-*co*-glycolic acid) (PLGA) polymer with an inherent viscosity of 0.17 dL/g and an average of 6000 g/mol molecular weight was purchased from Birmingham Polymers, Inc., Birmingham, AL. Recombinant DHBsAg and DHBcAg (Alberta strain-16) produced in yeast, were obtained from (Virexx Inc., Edmonton, AB, Canada). Poly(vinyl alcohol) (PVA) (molecular weight 31,000-50,000 g/mol, 89% hydrolyzed) was obtained from Sigma-Aldrich Canada, Ltd., Oakville, ON, Canada. Chloroform (HPLC grade) was purchased from Fisher Scientific International, Inc., Nepean, ON, Canada. Synthetic MPLA was kindly donated by Biomira, Inc. Edmonton, AB, Canada (30-32). (DHBcAg + MPLA)-

loaded PLGA nanoparticles were prepared by a water/oil/water (w/o/w) solvent evaporation method (33). Briefly, 200 μg of DHBcAg (0.8 mg/mL w/v; 250 μL) in a solution of PLGA in chloroform (100 mg in 400 μL) containing 100 μl of MPLA solution (2 mg/mL (w/v) in a chloroform/methanol mixture (4:1 (v/v)) was emulsified by sonication at level 4 for 15 second using a microtip sonicator (Model XL 2010; Heat Systems, Inc., Farmingdale, NY. This primary emulsion was then further emulsified in 2 mL 9% w/v polyvinyl alcohol (PVA) in phosphate buffered solution (PBS) by sonication at level 4 for 20 second to form a secondary emulsion. The resulting double emulsion was then added drop-wise to a stirring solution of PVA/PBS (9% (w/v); 8 mL). The emulsion was left to stir at room temperature for 3 hours to allow the evaporation of chloroform. The resulting nanoparticles were washed three times with sterile water (4 °C) and frozen immediately in a dry ice/acetone bath. They were then freeze-dried under vacuum for 3 days.

5.2.5 Measurement of particle sizes

The mean size diameter of the nanoparticles was determined by photon correlation spectroscopy (PCS) (Zetasizer 1000HS; Malvern Instruments, Ltd., Cirencester, UK) at 633 nm. Samples of the PLGA nanoparticles were suspended (3 mL; 0.07 μg/mL w/v) in 1 mM of NaCl for size measurements. CONTIN analysis was performed to measure the size and distribution of the particles.

5.2.6 Estimation of protein loading in nanoparticles

The micro-BCA assay (Pierce Biotechnology, Inc., Rockford, IL) was used to determine the quantity of recombinant DHBcAg loaded in the nanoparticle formulation. Protein was eluted from nanoparticles (10 mg) by stirring in 5% sodium dodecyl sulphate (SDS)/0.5 M NaOH v/v overnight. The sample was neutralized to pH 7 with 1 M HCl before analysis. The Micro BCA assay was performed in a 96-microwell plate (Gibco BRL), and the absorbance was measured at 562 nm by a scanning spectrophotometer (PowerWave X; Bio-Tek Instruments, Inc., Winooski, VT).

5.2.7 Serum DHBV DNA analysis - Real-time PCR assay

One week after each immunization, blood samples were taken from all ducks. Serum samples were also tested for the presence of DHBV DNA by dot blot hybridization and DNA extracted using the QIAmp DNA blood mini kit (QIAGEN). Viral DNA levels were then quantified by real-time PCR using the LightCycler (Roche, Indianapolis, IN). DHBV pAltD2-8 plasmid DNA with known amount of DHBV genomes was diluted with water to produce standards containing 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 genomes. As a negative control, template DNA was replaced by PCR-grade water. Amplification reactions were carried out in a total volume of 20 μL. Each reaction consisted of 10 μl of DNA (extracted from 10 μl of duck serum), 4 mM $MgCl_2$ (2.4 μl), 0.5 μM of primers (1 μl each) and 2 μL of FastStart DNA Master SYBR Green I and 3.6 μL of water. The primers were (forward: 5'TGAAGCAATCACTAGACC3') and (reverse: 5'ATGGTGGCTGCTCGAACT3'). The protocol required an initial denaturation for 10 min at 95°C and then 40 cycles of 5 s at 95°C, 10 s at 55°C, and 15 s at 72°C. The standard curve was generated by the Fit Points analysis method included in the LightCycler Data Analysis software, version 3.5.28, with the fit point number set at 2. Using this method, the log of the concentration of a dilution series of the standard or reference template DNA (DHBV pAltD2-8 plasmid) was plotted versus the cycle number at which the fluorescent signal increased above background or threshold (Ct value). DNA concentration was calculated by comparing the fluorescence of SYBR Green from samples, in the log-linear phase of amplification, to the known standards. The melting curve analysis program of the LightCycler was used to identify specific PCR products. Each PCR assay could detect at least 50 copies of viral target.

5.2.8 Anti-DHBcAg IgY antibody production- ELISA

Serum samples were analyzed for Anti-DHBs and Anti-DHBc antibodies by enzyme-linked immunosorbent assay (ELISA). Microtiter plates (96 well) were coated and incubated at 4°C overnight with 100 μL (1 μg/mL) DHBcAg or 100 μL (1 μg/mL) DHBsAg in 0.1 M $NaHCO_3$ (pH 9.6) at 37 °C overnight. The plates were washed with PBS containing 2% w/v bovine serum albumin (BSA). Serum samples diluted to 1/50 dilution with 2% BSA in PBS were added to the plates and incubated at 37 °C for 1 h. The plates were again washed three times with TPBS (0.05% v/v Tween 20 in PBS). The goat anti-duck IgG-HRP (Kirkegarrd Perry Labs Inc., Gaithersburg, MD) in 1/4000 dilution was added to the plates and incubated at 37 °C for 1 h. The plate was washed again with TPBS. Finally the bound antibodies were visualized by the addition of 100 μl of HRP substrate 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) substrate for colour development. An optical density (OD) reading at 405 nm was measured using a microplate scanning spectrophotometer (Molecular Devices, USA). Antibody titers were calculated relative to the pre-immune serum from the same animal. Data were expressed as the mean OD value of the samples minus the mean OD value of the normal sera from DHBV-negative normal ducks.

5.2.9 Statistical analysis

Comparisons were made between groups by a student's unpaired t-test. Statistical significance level was defined as $p<0.05$.

5.3 Results

5.3.1 Particle characterization

The mean volume diameters of the (DHBcAg+MPLA)-loaded PLGA nanoparticle and PLGA nanoparticle were 400 nm (polydispersity: 0.04), 400 nm (polydispersity: 0.14), respectively. DHBcAg encapsulation efficiency for the (DHBcAg + MPLA) PLGA nanoparticle was 81.6 % (± 9.18). The mean protein mass per dry weight of nanoparticles was equal to 0.163 % w/w for (DHBcAg +

MPLA) PLGA nanoparticles. An empirical vaccine dose with 10 mg of PLGA nanoparticles containing 15 μg of DHBcAg and approximately 9 μg of MPLA was used per immunization. An equal amount of a 15 μg dose of duck hepatitis B core protein (DHBcAg) was used for comparison.

5.3.2 Antigen-specific immune responses

5.3.2.1 Normal ducks

To evaluate the immunogenicity of the vaccine formulation in normal Pekin ducks, DHBV-uninfected Pekin ducks were immunized with PLGA nanoparticles containing DHBc and MPLA ((DHBcAg+MPLA) nanoparticles) once every 2 weeks for a period of 6 weeks total. The humoral immune responses were determined by enzyme immunoassays for specific IgY antibody against duck hepatitis B virus core protein (Anti-DHBc). Ducks immunized with (DHBcAg+MPLA) PLGA nanoparticles developed specific IgY antibody response against DHBcAg detectable in sera after the second vaccination (Figure 5-1 (A)). In contrast, ducks immunized with the same amount of DHBcAg protein without any immunomodulator or adjuvant did not produce a detectable antibody response, even following three immunizations of the protein vaccine (Figure 5-1 (B)). No anti-DHBc IgY response was detected in the control group receiving PLGA nanoparticles (Figure 5-1 (C)).

5.3.2.2 Congenitally DHBV-infected ducks

To determine the humoral immune response to the vaccine formulation in congenitally DHBV-infected ducks, the infected ducks were immunized with (DHBcAg+MPLA) nanoparticles once every 2 weeks for a period of 6 weeks total. Anti-DHBc IgY was induced in ducks immunized with the (DHBcAg+MPLA) nanoparticles (Figure 5-2(A)). These antibodies could be then boosted by further immunizations in the majority of the ducks. In contrast, in sera of the congenitally DHBV-infected ducks receiving control vaccine PLGA nanoparticles, no anti-DHBc could be detected (Figure 5-2 (C)). Similarly, the group of ducks receiving DHBcAg alone developed no anti-DHBc IgY antibody (Figure 5-2(B)).

5.3.2.3 Experimentally DHBV-infected ducks

To determine the humoral immune response to the vaccine formulation in experimentally DHBV-infected ducks, infected ducks with persistent DHBV infection were immunized with (DHBcAg+MPLA) nanoparticles once every two weeks for a period of 6 weeks total. The ducks immunized with (DHBcAg+MPLA) nanoparticles had developed IgY antibody to DHBc after the second immunization dose (Figure 5-3). Serological profile of the ducks receiving either DHBcAg alone or PLGA nanoparticles was not determined due to insufficient amounts of sera collected from these ducks.

5.3.3 Virological outcome

To evaluate the correlation of vaccine-induced immune responses to inhibition of DHBV replication, serum DHBV DNA (viral load) was determined by real time polymerase chain reaction (PCR) before and during the vaccine trial. Two types of chronic infection models were used including the congenitally DHBV-infected and experimentally DHBV-infected ducks. The congenitally DHBV-infected ducks immunized with (DHBcAg+MPLA) nanoparticles had a slight reduction of viral load over time (Figure 5-4 (A)) except for duck 83 who had an increase of DHBV DNA at the end of the study. A slight reduction of viral load was also found in ducks receiving DHBcAg alone (Figure 5-4 (B)). However, a 3-log reduction of viral load was noted in one of the ducks (duck 667) from the control ducks receiving the PLGA nanoparticles (Figure 5-4 (C)). The reduction of viral load between the test groups and the control were not statistically different ($p = 0.05$).

From the experimental DHBV-infected ducks, 5 out of 6 ducks immunized with (DHBcAg+MPLA) nanoparticles had a progressive reduction of viral load over time (Figure 5-5 (A)). Duck #131 from the (DHBcAg+MPLA) nanoparticles immunized group had a more than a 3-log reduction of viral load at the end the treatment. Ducks immunized with DHBcAg alone had a variable level of viral load over the 42 days vaccine trial (Figure 5-5 (B)). A 1 or 2-log reduction of viral load was noted in control groups receiving PLGA nanoparticles (Figure 5-5 (C)). Overall, there is no statistically difference in the change of viral load between the test groups and the control ($p=0.05$).

5.4 Discussion

PLGA nanoparticles containing MPLA and DHBcAg were formulated as a DHBV vaccine. To test the ability of this vaccine formulation to stimulate specific immune response in ducks, Pekin ducks were immunized with this vaccine. The elicited antigen-specific antibody responses were monitored during the vaccination trial. Following a second vaccination with (DHBcAg+MPLA) nanoparticles, anti-DHBc IgY could be detected in the sera of Pekin ducks. By contrast, the same amount of DHBcAg injected alone did not yield detectable core-specific antibody responses in ducks. These results suggest that an MPLA-adjuvanted DHBcAg vaccine delivered by PLGA nanoparticles is capable of eliciting and enhancing core-specific antibody responses in ducks. The adjuvant effects of PLGA particles and MPLA had been demonstrated for various antigens in murine model (34-37). This is the first study to demonstrate the immunopotentiating effect of PLGA nanoparticles and MPLA to the incorporated antigen in ducks

Hatchling ducks infected with DHBV and congenitally DHBV-infected ducklings represent the horizontal and vertical mode of transmission, respectively. They serve as DHBV-infection models for the study of chronic hepatitis B virus infection. A lack of immune response or an immune hyporesponsiveness to DHBV has been described to contribute to the persistence of viral infection in the early or congenital infection (38). Immune tolerance in chronically DHBV-infected ducks was characterized by persistent viral load and the lack of anti-DHBs and anti-DHBc antibody responses (39, 40).

This is different from the hepatitis B virus (HBV)-infecting humans, where anti-HBc antibodies are present in chronic HBV carriers (41).

Several lines of evidence suggests that hepatitis B virus (HBV) core antigen (HBcAg)-specific CD4+ T-cell responses play an important role in the control of HBV infection (42-46). Although core antigen constitutes the internal structure of the hepatitis B virus, immunization of chimpanzees with HBcAg generated protective immunity against viral infection (47, 48). Similar protective effect had been demonstrated in woodchucks immunized with woodchuck hepatitis B virus core antigen (WHcAg) (49, 50). Furthermore, immunization of chronically WHV-infected woodchucks with WHcAg resulted in induction of specific T cell responses and a control of viral infection (51). Bone marrow containing hepatitis B core-specific T cells transferred to a HLA-matched recipient resulted in a resolution of chronic hepatitis B infection (52). Thus, hepadnaviral core antigen represents an important target for therapeutic vaccine.

Therapeutic vaccines for chronic hepatitis B were designed to stimulate effective virus-specific immune responses in the infected hosts to eradicate viral infection. PLGA nanoparticles containing DHBcAg and MPLA was proposed in this study as a DHBV vaccine for the treatment of chronic DHBV. We hypothesized that immunization with such particulate vaccine formulation is effective in inducing antiviral immunity for the elimination of DHBV infection. The results showed anti-DHBc IgY could be induced by immunization with (DHBcAg+MPLA)-loaded nanoparticles and boosted by booster doses, suggesting that immune tolerance to viral antigen in chronically DHBV-infected ducks can be overcome by the particulate vaccine formulation.

A fluctuation of viral load between 10^3 to 10^6 DHBV DNA copies in 10 μl sera was observed in both congenitally and experimentally DHBV-infected ducks. Such a wide range of variations in viral load over time in chronically DHBV-infected ducks interferes with the assessment of the vaccination results. Therefore, the immunostimulatory effects of the vaccine formulation on inhibition of DHBV replication were inconclusive based on the results in this study. Failure of this vaccine formulation to reach virus elimination may be due to the fact that insufficient antiviral immune responses were induced. Vaccine dosage, timing and frequency of immunization in relation to the induction of immune responses in ducks have not been fully explored. The optimization of these factors is needed in order to generate an effective immune response for viral elimination.

Alternatively, general CD4$^+$ T cell dysfunctions have been found in patients with chronic hepatitis B (53). An altered T helper 0/T helper 2 cytokine profile in response to viral antigen was produced in HBV carriers (54). Functionally defective dendritic cells in the peripheral blood monocytes of patients with chronic HBV infection have also been described (55-57). It is unclear whether dysfunctional CD4$^+$ T lymphocytes and/or impaired dendritic cells were present in ducks with chronic DHBV infection, which may also account for the failure of the vaccine to eliminate virus.

Cell mediated immunity (CMI) is a critical factor in determining the clinical outcomes of hepatitis B infection. It has been proposed that an effective therapeutic vaccine for chronic hepatitis B should induce sufficient quantity and quality of cytotoxic T lymphocytes and CD4$^+$ T lymphocytes with a predominant Th1-cytokine profile (58). The release of antiviral cytokines IFN-γ, and TNF-α by cytotoxic T lymphocytes, dendritic cells and macrophages is an important mechanism in the non-cytolytic hepatitis B viral clearance (59-61). Monitoring of the cellular immune responses and the cytokine profile during immune therapy will provide more informative data on the immune-mediated viral clearance effects induced by the vaccine. Furthermore, since cccDNA is the replicative template for the virus, liver biopsy samples should be collected in future studies to assess changes in the cccDNA during vaccine trial.

Taken together, this study demonstrated the safety of PLGA nanoparticle formulation as a vaccine in ducks and the immunopotentiating effect of PLGA nanoparticles and MPLA as vaccine adjuvants for DHBcAg. To fully exploit the therapeutic potential of (DHBcAg+MPLA) PLGA nanoparticles, further studies in optimizing the vaccine dose, the route and frequency of immunization in ducks are warranted.

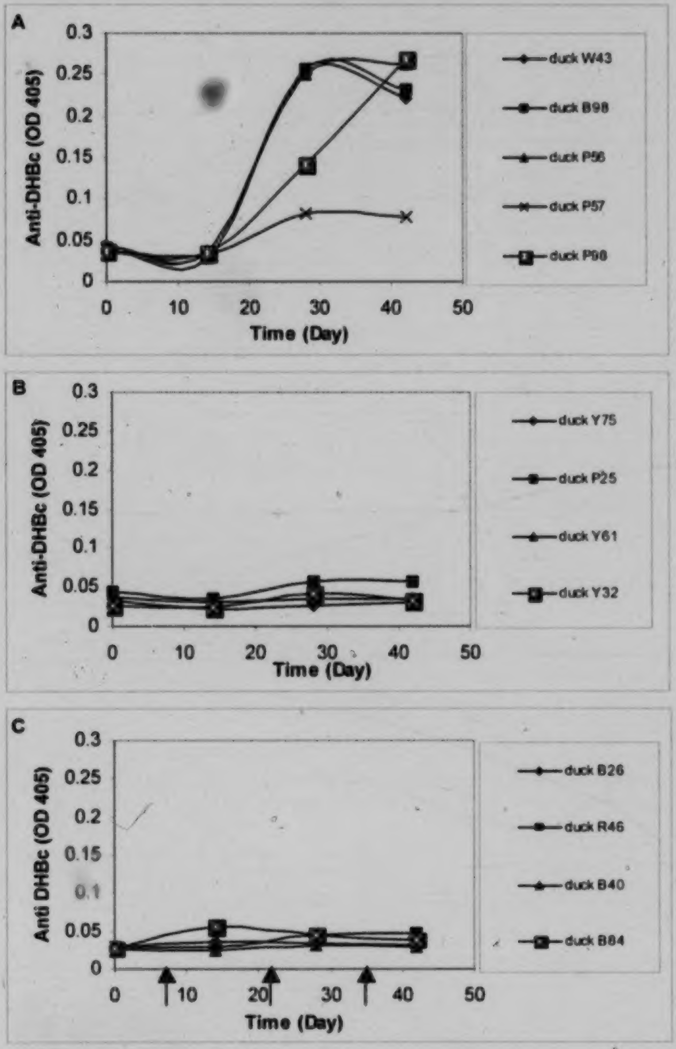

Figure 5-1. Anti-DHBc IgY responses of normal Pekin ducks immunized with either (A) (DHBcAg+MPLA) nanoparticles, (B) DHBcAg or (C) PLGA nanoparticles control, on three occasions day 7, day 21 and day 35 (marked with arrows on the x-axis). Serum samples were collected seven days prior to the immunization (day 0) and seven days after each immunization. Each line represents the anti-DHBc response of an individual duck to the treatment over time. Serum samples were used at 1/50 dilution.

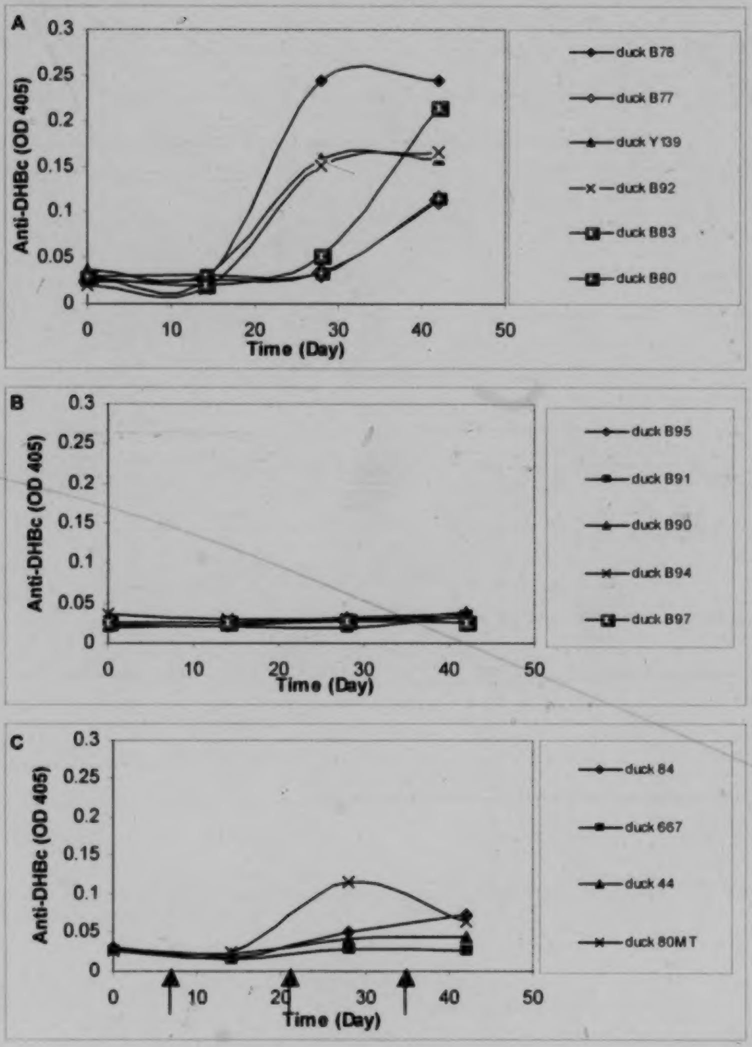

Figure 5-2. Anti-DHBc IgY responses of congenitally DHBV-infected ducks immunized with either (A) (DHBcAg+MPLA) PLGA nanoparticles, (B) DHBcAg or (C) PLGA nanoparticles control, on three occasions day 7, day 21 and day 35 (marked with arrows on the x-axis). Serum samples were collected seven days prior to the immunizations (day 0) and seven days after each immunization. Each line represents the anti-DHBc response of an individual duck to the treatment over time. Serum samples were used at 1/50 dilution.

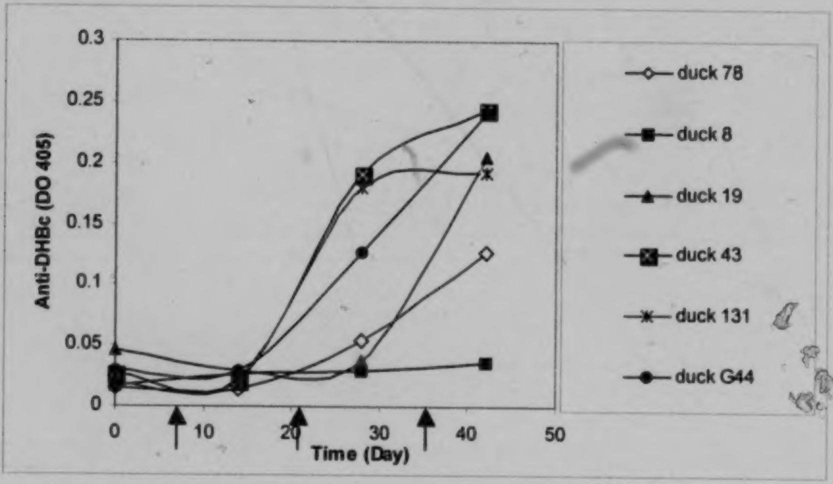

Figure 5-3. Anti-DHBc IgY response of experimentally DHBV-infected ducks immunized with (DHBcAg+MPLA) PLGA nanoparticles on three occasions day 7, day 21 and day 35 (marked with arrows on the x-axis). Serum samples were collected seven days prior to the immunizations (day 0) and seven days after each immunization. Each line represents the anti-DHBcAg response of an individual duck to the treatment over time. Serum samples were used at 1/50 dilution.

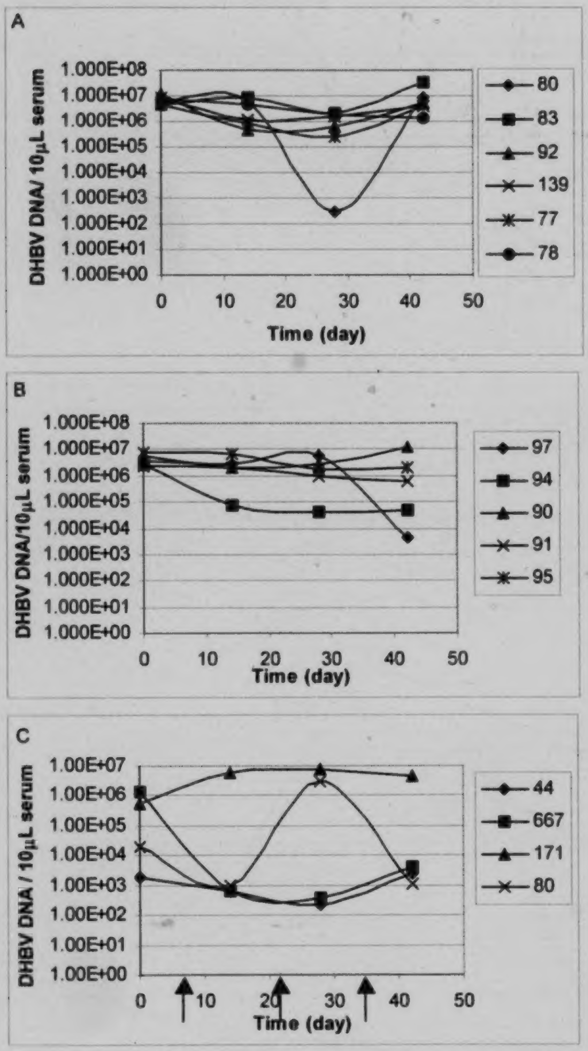

Figure 5-4. Serological profiles of DHBV DNA of congenitally DHBV-infected ducks immunized with either (A) (DHBcAg+MPLA) PLGA nanoparticles, (B) DHBcAg or (C) PLGA nanoparticles control on day 7, day 21 and day 35 (marked with arrows on the x-axis). Serum samples were collected seven days prior to the immunization (day 0) and seven days after each immunization.

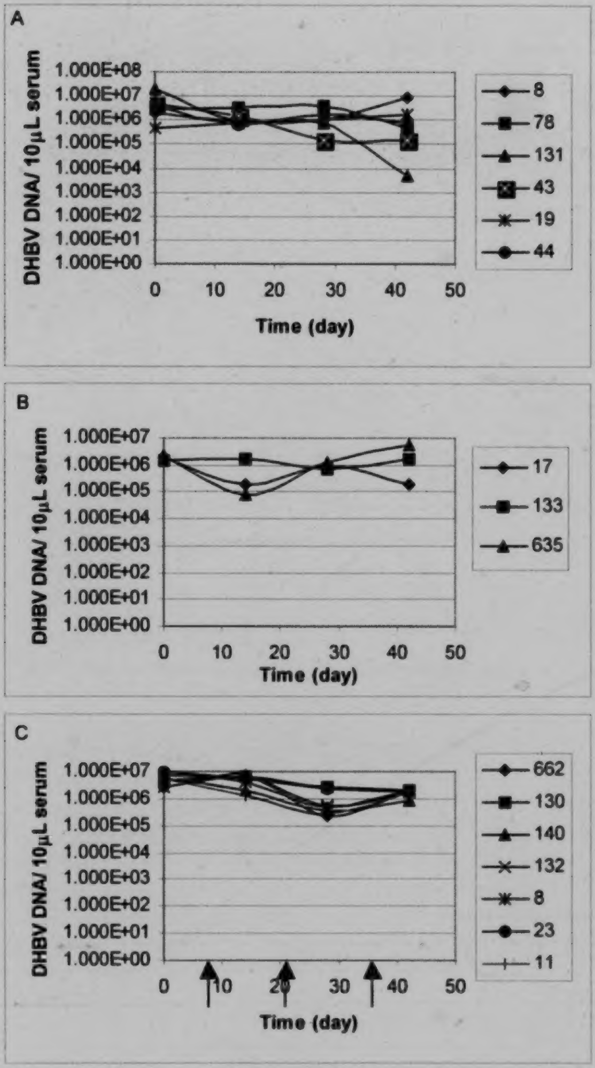

Figure 5-5. Serological profiles of DHBV DNA of experimentally DHBV-infected ducks immunized with either (A) (DHBcAg+MPLA) PLGA nanoparticles, (B) DHBcAg or (C) PLGA nanoparticles control on day 7, day 21 and day 35 (marked with arrows on the x-axis). Serum samples were collected seven days prior to the immunization (day 0) and seven days after each immunization.

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Chapter 6

General Discussion and Conclusions

6.1 Discussion

Currently, more than 400 million people in the world are chronically infected with hepatitis B virus (HBV), in spite of the presence of an effective prophylactic vaccine since 1982 (1). A significant number of chronic HBV carriers can eventually develop serious complications such as liver cirrhosis and hepatocellular carcinoma (2). A collateral impact is that while infected, chronic HBV carriers are the sources of HBV infection and transmit HBV to healthy uninfected individuals. Taken together, chronic HBV infection represents a major global public health concern, especially in the developing countries of Asia and Africa, where the majority of the chronic HBV-carriers reside. To date there is still no effective curative therapy for these patients. The treatment of chronic HBV infection by antiviral agents like type-1 interferon was unsatisfactory due to their low efficacy, considerable side effects and high costs (3). Nucleoside analogues such as lamuvidine are effective antiviral reagents, but mutations in the viral genome with long-term use are frequently associated with a recurrence of HBV replication. An alternative approach to HBV treatment is urgently needed.

Human HBV is a DNA virus that causes both acute and persistent liver infections. A vigorous, polyclonal and multispecific cytotoxic (CTL) and helper T (Th) cell responses to HBV were readily detectable in the peripheral blood of patients with acute self-limited hepatitis B, whereas weak, narrowly focused or undetectable HBV-specific T cells were found in patients with chronic infection (4). The strength of antiviral immunity was clearly associated with the infection outcome, thus providing the basis for immunotherapy.

Virus-specific CD4$^+$ T cells had been shown to play a major role in successful viral clearance in acute hepatitis B (2, 5). While it is important to induce cytotoxic T lymphocytes (CTL) for viral elimination, the induction of CD4$^+$ T cell is instrumental to the success of viral clearance as it has been shown to play a pivotal role in the maintenance of effective antiviral CD8 T cell responses and the development of functional CD8$^+$ T cell memory (6-8). A preferential HBcAg-specific Th1 lymphocyte response was activated in patients with acute self-limited hepatitis B infection (4) whereas a weak HBcAg-specific immune reaction with a mixed Th1/Th2 cell type was found in chronically HBV-infected patients (5), thus suggesting the importance of core-specific immune response of Th1 type that is associated with the ability for viral clearance. Therefore, it is hypothesized that a preferential induction of a HBcAg-specific Th1 type T-helper response with production of gamma interferon (IFN-γ) could facilitate eradication of chronic HBV infection.

An improved understanding of the underlying mechanisms involved in the presentation and generation of T cell immune response contributes to the rational design of a better vaccine for immunotherapy with PLGA nanoparticle formulation. Together with antigen, an immunomodulator, monophospholipid A were encapsulated in PLGA nanoparticles for vaccine therapy. Since antigen-presenting cells play a major role in initiating immune responses, PLGA nanoparticles served as a vaccine carrier, were used to target and deliver antigen to antigen-presenting cells such as dendritic cells and macrophages. The adjuvant activity of PLGA nanoparticles for the delivery of peptide, protein or DNA vaccine, has been demonstrated in numerous studies in the murine model (9-11). Targeted delivery of vaccine to dendritic cells has important implication as dendritic cells provide a critical link between the innate and adaptive immune responses (12).

The toll-like receptors (TLRs) on the surface of the antigen-presenting cells allow them to detect the pathogen associated molecular patterns (PAMPs) on microbes efficiently, triggers antimicrobial host defence mechanisms, and activates the necessary signal to initiate adaptive immune responses (13). The inherent particulate nature of PLGA nanoparticles promotes antigen delivery into APC for presentation to T cells to generate 'signal 1'(antigen recognition) (14). MPLA released from the vaccine formulation, signalling through TLR-4 (15), alerts the innate immunity by triggering local inflammatory responses to stimulates APC to express the co-stimulatory molecules 'signal 2'. This events lead to the induction of adaptive immune response in the context of "infectious non-self" (16) and amplify the signals generated by antigen-specific recognition events.

Previous studies on protein antigen loaded in PLGA nanoparticles revealed the ability of the vaccine formulation to elicit antibody response, CTL and T cell proliferative responses to the encapsulated antigen (17-19). In Chapter 2 a hepatitis B therapeutic vaccine candidate, hepatitis B core (HBcAg) was incorporated into PLGA nanoparticles with or without MPLA to evaluate the specificity, strength and the type of T helper responses elicited by the nanoparticle formulations in the murine model. The results revealed that HBcAg protein loaded PLGA nanoparticles elicited antigen-specific T cell responses with a cytokine profile suggestive of a Th1 response. Moreover, the incorporation of MPLA to the vaccine formulation further enhanced the Th1 response as evidenced by the increased T cell proliferative responses and IFN-γ production. More importantly, the level of HBcAg-specific IFN-γ production could be amplified to a greater extent by a booster immunization with the nanoparticle formulation containing HBcAg and MPLA. Hence, it was concluded that co-delivery of HBcAg and MPLA in PLGA nanoparticles is capable of enhancing HBcAg-specific Th1 immune response and promoting IFN-γ production. The HBcAg release rate from the nanoparticles with MPLA was not significantly different from the formulation without MPLA; therefore, the observed difference in the immune activities cannot be attributed to the protein release rate.

With the ability to induce a robust Th1 favoring immune response with a high level of IFN-γ production, the co-delivery of HBcAg and MPLA by the biodegradable PLGA nanoparticles in a single formulation presents as a promising therapeutic vaccine strategy for the treatment of chronic hepatitis B infection. The production of high level of IFN-γ is particularly important as it has a major role in mediating non-cytolytic viral inhibition and reducing cccDNA (20), which may lead to the eradication of chronic HBV infection.

The murine model is useful for evaluating the immunopotentiating effects of various vaccine formulations. However, the ultimate question of whether vaccination with the nanoparticle formulation confers protective immunity or if it is effective for therapy of chronic hepatitis B cannot be answered in a mouse model that is not susceptible to HBV infection. Duck hepatitis B virus (DHBV) infecting Pekin duck is a more suitable model for study of the effectiveness of vaccine therapy. DHBV is closely related to the human HBV, the chronic infection of ducks resemble many aspects of what occurs in the chronic HBV carrier state, therefore allowing the use of the duck for testing the efficiency of antiviral treatments (21).

The drawback of using DHBV model is that the duck immune system has not been as extensively explored as chicken or mammalian immune systems. The lack of reagents to identify the cell surface markers present on the duck immune cells hampers the development of methods to purify and characterize the individual immune cell types in Pekin ducks. The long-term goal of the continual research in the lab is to characterize the duck immune system for vaccine testing. As MHC class II and invariant chains are expressed primarily by APC, the cloning and sequencing of the duck homologues of MHC class II DRA and invariant chain were performed to facilitate the identification of duck APC. The DNA sequence information of duck MHC class II DRA and invariant chain were elucidated in Chapter 3. Specific primers for MHC class II DRA and invariant chain were designed and used to examine the tissue expression levels in APC-rich organs. Similar to their mammalian counterpart, the expression of duck MHC class II DRA and invariant chain were particularly enriched in the lymphoid organs.

Appropriate antigen uptake for processing and presentation by antigen presenting cells such as macrophages, dendritic cells and B cells is the prerequisite for stimulating an effective immune response (14). PLGA particles formulated as antigen delivery system are effective in potentiating the immune response to the incorporated antigen in murine model (22-24). The uptake of PLGA particles by duck macrophages was examined in Chapter 4. Phagocytosis of PLGA particles by duck macrophages is dependent on the size of particles. PLGA nanoparticles of an average of 687nm in diameter were phagocytosed by duck macrophages whereas PLGA microparticles greater than 14 μm were not.

The presence of lipopolysaccharide (LPS) increased the efficiency of PLGA particle uptake by duck macrophages. The presence of LPS also triggered the production of nitric oxide by duck macrophages in a dose and time-dependent manner. Duck macrophages capable of phagocytosing and release inflammatory mediators display innate immunity to eliminate foreign substances similar to that of mammalian cells (25).

In mammalian cells, scavenger receptors are found on the surface of phagocytes (26). They bind to LPS on the bacterial cell wall to promote the attachment of microorganisms to phagocytes for subsequent engulfment and destruction. LPS is also recognized by toll-like receptors (TLR-4) and CD14. Binding of LPS to these receptors stimulates the production and secretion of cytokines that are crucial to initiating innate immunity and adaptive immunity (27). Although these pattern-recognition receptors have not been identified in ducks, similar mechanisms are likely involved during the recognition of conserved microbial components such as LPS in ducks, leading to the promotion of innate immune defense such as phagocytosis.

Isolation of duck APC will aid in the characterization of the cellular immune system in ducks. PLGA particles containing tetramethylrhodamine-labeled dextran (TMR), a fluorescence probe, were used to identify and isolate phagocytic cells. The expression of MHC class II DRA and an invariant chain were found in this phagocytic population and therefore suggesting that these duck phagocytic cells are capable of processing and presenting exogenous-derived antigen. The results of this study also provide direct evidence to support the use of PLGA nanoparticles to target and deliver antigen into duck APC, which may be critical for the adjuvant effects of PLGA nanoparticles as an antigen delivery system.

Based on the promising results observed in mice from the earlier vaccination studies with PLGA nanoparticle formulations, a preliminary study was undertaken to investigate the therapeutic efficiency of nanoparticle formulation on chronic hepadnavirus infection (Chapter 5). The duck hepatitis B virus model was used for vaccine therapy studies. PLGA nanoparticles containing MPLA and DHBcAg were formulated as a DHBV vaccine. The studies in chapter 5 demonstrated the ability of this vaccine to induce anti-DHBc IgY antibody response in normal ducks. The same dose of DHBcAg used in the immunization did not result in the production of antibody responses. This is the first study demonstrating that the immunopotentiating effect of PLGA nanoparticles and MPLA to the incorporated antigen in ducks PLGA. These observations suggest the potential of MPLA-adjuvanted DHBcAg vaccine delivered by PLGA nanoparticles as a therapeutic vaccine for treatment against hepadnaviral infections.

Cell mediated immunity (CMI) is a critical factor in determining the clinical outcomes of hepatitis B infection. Antiviral cytokines such as IFN-γ, and TNF-α also play important roles in the elimination of hepadnaviruses. In the future studies, monitoring of the cellular immune responses and the cytokine profile during immune therapy is needed. This will provide important information to assess the immune-mediated viral clearance effects induced by the vaccine.

To determine the ability in overcoming the immune non-responsiveness to persistent DHBV infection with this vaccine formulation, congenitally and experimentally infected ducks were used in the vaccine trial. Although there was a general reduction of viral load in ducks receiving the vaccine, the overall viremic changes in the test groups were not significantly different from the control. The normal wide range of viremic fluctuation in DHBV-infected ducks made it difficult to assess the outcome of vaccine therapy. It is worth noting that the chronically DHBV-infected ducks developed anti-DHBc in the groups receiving nanoparticle formulation. This suggests that the immune tolerance to DHBc antigen in the chronically DHBV-infected ducks was overcome by the vaccine therapy.

The outcome of vaccine therapy was usually heterogeneous in both human and murine hepatitis B virus (HBV)-carriers (28, 29). It has been proposed that the function of dendritic cells provides prognostic value to predict the outcome of vaccine therapy (30). There are functional defects of dendritic cells and a general altered balance of Th1/Th2 response observed in some patients with chronic HBV infection, which may contribute to the variable responses to therapeutic vaccine immunization (31-33).

The physiochemical parameters of the PLGA nanoparticles were known to affect the immunological responses in mice (34-36). More work is needed to delineate methods to optimize both uptake of PLGA nanoparticles and immune activation by the vaccine formulations in DHBV-infected ducks, which may lead to effective immune responses for viral inhibition.

The use of PLGA nanoparticle formulation as a vaccine delivery system for enhancing immune responses in duck appears promising. Increased endeavour to increase our knowledge of the immune system of Pekin ducks will be integral to the success of vaccine therapy in DHBV model. The study of vaccine in DHBV model should prove valuable for refining the vaccine formulations and their design to produce an effective immunotherapy for chronic hepatitis B in the near future.

6.2 Conclusions

1) Co-delivery of HBcAg with MPLA in PLGA nanoparticulate formulation elicited robust antigen-specific cell-mediated immune responses specifically T helper type 1 (Th1) response and generated a high level of IFN-γ production.
2) The cDNA clones of duck MHC class II DRA and invariant chain homologues were sequenced and characterized.
3) Duck macrophages, an important professional antigen-presenting cell, are capable of phagocytosing PLGA nanoparticles. Duck macrophages with phagocytic properties can be identified and separated using PLGA nanoparticles containing a fluorescent probe.
4) PLGA nanoparticle formulation was capable of inducing and enhancing humoral response to the encapsulated antigen in Pekin ducks.
5) MPLA-adjuvanted DHBcAg delivered by PLGA nanoparticles was able to produce DHBc-specific IgY antibody in the DHBV-chronically infected ducks, suggesting that immune tolerance to viral antigen in these ducks could be overcome by vaccine therapy.

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